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Muğla Sıtkı Koçman University, Turkey
r.mammadov@natprobiotech.com
rmammad@yahoo.com

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m.turan@natprobiotech.com
muratturan077@gmail.com

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Phenolic Compounds Screening and Potential of Larvicidal Activity of Water Extract of *Cyclamen cilicium* Boiss. & Heldr.

Murat Turan[1*], Ramazan Mammadov[2]

[1] Department of Molecular Biology and Genetics, Faculty of Science, Erzurum Technical University, Erzurum, Turkey
[2] Department of Molecular Biology and Genetics, Faculty of Science, Muğla Sıtkı Koçman University, Muğla, Turkey

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Abstract

This study was designed to phenolic compound analysis with UPLC-ESI-MS/MS, larvicidal (against *Musca domestica* and *Culex pipiens*) activities with fresh and underground parts of water extract of *Cyclamen cilicium* Boiss. & Heldr. Thirty one standard phenolic compounds were used in UPLC-ESI-MS/MS analysis, and ferulic acid was found to value 4483.34 mg/kg as the major compound. The fresh part was found a potential larvicidal activity with 33.33 ± 4.81 % against *M. domestica* and the fresh part was found potential larvicidal activity than underground part with 0.43 ± 0.09 mg/mL, LC_{50} against *Cx. pipiens.* These results about *C. cilicium* were shown as a potential biolarvicidal potential and can be used in the pharmaceutical, agricultural industry.

Corresponding Author:
Murat Turan, Department of Molecular Biology and Genetics, Faculty of Science, Erzurum Technical University, Erzurum, Turkey, muratturan077@gmail.com
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1. INTRODUCTION

Many insects are mechanical vectors, carrying many important diseases (Cossetin *et al*., 2021). Recently, diseases transmitted by vector organisms are increasing all over the world (Semiatizki *et al*., 2020). Housefly [*Musca domestica* L. (Diptera: Muscidae)] and mosquito [*Culex pipiens* L. (Diptera: Culicidae)] are cosmopolitan vectors and cause a serious threat to human health and livestock (Nisar *et al*., 2021). Mosquitoes are among the most important arthropod groups in terms of human and animal disease entomology and are the mechanical carriers of very important diseases such as malaria, yellow fever, dengue, filariasis, encephalitis (Vatandoost et al., 2012). Malaria is one of the global health problems in sub–Saharan Africa and has been increasing alarmingly over the past decade (Nhaca *et al*., 2020). In 2018, the mortality rate of children under 5 from malaria was 67% in sub–Saharan Africa (WHO Global, 2019). House flies are mechanical vectors that carries more than 100 different pathogens (bacteria, fungi, viruses, etc.), can easily live on the ground in the settlements of people and animals and can infect them (Khamesipour *et al*., 2018; Mahyoub, 2021). House flies have important contributions to the spread of various infectious diseases such as cholera, typhoid, dysentery, tuberculosis (Chintalchere *et al*., 2013). Keeping mechanical vectors such as mosquitoes and house flies under control is necessary to prevent the spread of many important diseases. The most used chemicals for this are organochlorines, organophosphates, carbamates (Scott *et al*., 2000). Vector creatures have a very high rate of resistance against chemical insecticides. A study conducted in the Manhiça Prefecture, Maputo State, Mozambique found that *Anopheles funestus* developed resistance to chemical drugs and 90 % of mosquitoes survived under the influence of deltamethrin or lambda-cyhalothrin (Glunt *et al*., 2015). For this, it is necessary to increase the chemical rate or to try other chemicals that are less likely to

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create resistance. The chemicals used trigger potential toxicity in humans and animals (Kaufman *et al*., 2001; Shono *et al*., 2004; Nisar *et al*., 2021). Potential larvicidal / insecticidal experiments of plant extracts are increasing day by day as they are obtained from edible sources, biodegradable, do not leave residue, and are less toxic to humans and animals (Rodrigues *et al*., 2021). Secondary metabolites in plant content can be used as larvicides, insecticides, repellents, ovipositional attractants, and can be used as alternative and less resistant larva control agents (Kamaraj and Rahuman, 2010). *Cyclamen* genus species belonging to the Primulaceae family is represented by 11 species and 12 taxa in Turkey (Güner *et al*., 2012). Several *Cyclamen* species have some investigations that contain some triterpene saponins, glycosides, and phenolic components (Sarikurkcu, 2011; Metin *et al*., 2013).

There are no reported studies on the phytochemical composition and larvicidal activities of *C. cilicium*. Therefore, this study aimed to evaluate the chemical compounds screening and the larvicidal activity against *Musca domestica* L. and *Culex pipiens* L. of fresh and underground parts of *C. cilicium* extracts of water solvent. This study's results guide the further applications of the above and underground parts of *C. cilicium* in nutraceutical and pharmaceutical production.

2. MATERIAL and METHODS

2.1. Plant Materials and Extract Preparation

Cyclamen cilicium Boiss. & Heldr. was collected at 938 m altitude in September 2018 during the flowering period from Antalya province in Turkey. The plant material was identified by Dr. Olcay Düşen and stored with voucher specimens (Herbarium No: 1004 M. Turan) at PAMUH in Pamukkale University, Denizli, Turkey. At room temperature, dried fresh and tuber parts of *C. cilicium* were cut and were extracted with water as solvent. It was kept in a shaking water bath for 6 hours and filtered through Whatman paper, and the solvent was added again (Memmert WNB 14). After filtration, water was evaporated (IKA RV 10 and Labconco Freezone 6). Extracts were kept at -20 ^{o}C (Yılmaz *et al*., 2019).

2.2. Analysis of Phenolic Compounds by UPLC- ESI-MS/MS

Analysis of phenolic compounds in UPLC-ESI-MS/MS with standards has been performed according to the method of Kıvrak and Kıvrak (2017). A total of 31 phenolic compounds were identified based on retention times and mass spectra of commercial standards.

2.3. Assay of Larvicidal Activity to Housefly (*Musca domestica*) Larvae

Larvicidal plant extracts were investigated by modifying the Çetin et al. (2006) method to housefly (*M. domestica*) larvae. Houseflies (*M. domestica*) used in the assays were used as the 365th generation of the World Health Organization strain. The second-, third instar larvae were used for bioassays. The larvae were reared at 16:8 light/dark photoperiod, 50 ± 10 % RH, and 26 ± 2 ^{o}C. The study was carried out in 2 doses (1 and 5 mg/mL). Milk and sugar were used for *M. domestica* culture, and the mixture was prepared as 1:3 and 50 g. After 24-36 hours, the eggs started to open, and the larvae emerged. 25 house flies were taken from their eggs and transferred to the medium containing extract and moisture. The larvicide effect was recorded within three weeks. The larvicidal effect was performed in the 16:8 light/dark photoperiod at 26 ± 2 ^{o}C in a laboratory setting.

2.4. Assay of Larvicidal Activity to Mosquito (*Culex pipiens*) Larvae

Larvicidal activity against mosquito (*Cx. pipiens*) larvae of the extracts were investigated according to the method of Oz et al. (2013). Mosquito (*Cx. pipiens*) used in the assays were collected from a pool in August 2019. The second-third instar larvae were used for bioassays. Extract solutions dissolved in water at a concentration of 0.1-1 mg/mL are added to 100 mL of

distilled water. Then 12 larvae are added. Larvae that died after 24, 48, and 72 hours in a 26 ± 1 ºC environment in the 12:12 (L:D) photoperiod were counted.

2.5. Statistical Analysis

All assays were performed in 3 replicates. The results were analyzed using the Statistical Package for Social Sciences (SPSS) statistical software (2017). Significant differences among groups were identified by one-way analysis of variance (ANOVA) with Duncan's multiple range test, setting $p \leq 0.05$ as the level of significance $LC_{50(min)}$, LC_{50}, $LC_{50(max)}$, $LC_{90(min)}$, LC_{90}, $LC_{90\ (max)}$ was made by Probit analysis in STATPLUS (2015) program in larvicidal activity assays.

3. RESULTS and DISCUSSION

Phenolic compounds determined by UPLC-ESI-MS/MS from *C. cilicium* are given in Table 1. Thirty one phenolic compounds were identified according to retention times and mass spectra of commercial standards. In our study, the ferulic acid compound was found to 4483.34 mg/kg as the major compound. Total ion chromatograms (TIC) are shown in Figure 1 as the content of phenolic compounds in *C. cilicium* is abundant. Ferulic acid has a strong ability to scavenge free radicals. Therefore, it is a useful chemical component in preventing important diseases such as cancer caused by oxidative stress. Ferulic acid has a skin protective effect thanks to its ability to absorb UV high light (Zhao and Moghadasian, 2008; Tuncel and Yılmaz, 2010). The reason for the high antioxidant activity of *C. cilicium* is thought to be the high contribution of vanillic acid found in HPLC analysis.

Table 1. Phenolic compounds of *C. cilicium* by UPLC-ESI-MS/MS (mg/kg).

	1	2	3	4	5	6	7	8	9	10	11
F. P.	ND	ND	38.77	5.98	ND	25.72	1.52	ND	ND	ND	1957.01
U. P.	ND	ND	10.7	0.78	ND	4.46	0.69	ND	ND	ND	199.9
	12	13	14	15	16	17	18	19	20	21	22
F. P.	1230.51	1804.91	1256.49	ND	18.12	273.75	4483.34	ND	ND	ND	ND
U. P.	21.93	22.0	463.49	ND	10.43	1.83	200.34	ND	ND	ND	ND
	23	24	25	26	27	28	29	30	31		
F. P.	ND	ND	ND	ND	ND	ND	ND	ND	378.85		
U. P.	ND	ND	ND	ND	ND	ND	ND	ND	45.23		

*F.P.: Fresh Part, U.P.: Underground Part. **1: Genistein, 2: Galanthamine, 3: Quercetine, 4: Pyrocatechol, 5: Pyrogallol, 6: 4-Hydroxy-benzoic acid, 7: 3-4-dihydroxy benzaldehyde, 8: *trans*-cinnamic acid, 9: Vanillin, 10: Gentisic acid, 11: Protocatechuic acid, 12: *p*-Coumaric acid, 13: *trans*-2-hydroxy cinnamic acid, 14: Vanillic acid, 15: Homogentisic acid, 16: Gallic acid, 17: Caffeic acid, 18: Ferulic acid, 19: Syringic acid, 20: Resveratrol, 21: Chrysin, 22: Apigenin, 23: Naringenin, 24: Kaempferol, 25: Luteoline, 26: Catachin hydrate, 27: Epicatechin, 28: Hesperitin, 29: Myricetin, 30: Catechin gallate, 31: Rutin, ND: not detected.

Figure 1. Total ion chromatograms of ferulic acid compound of fresh (a) and underground (b) parts of *C. cilicium*.

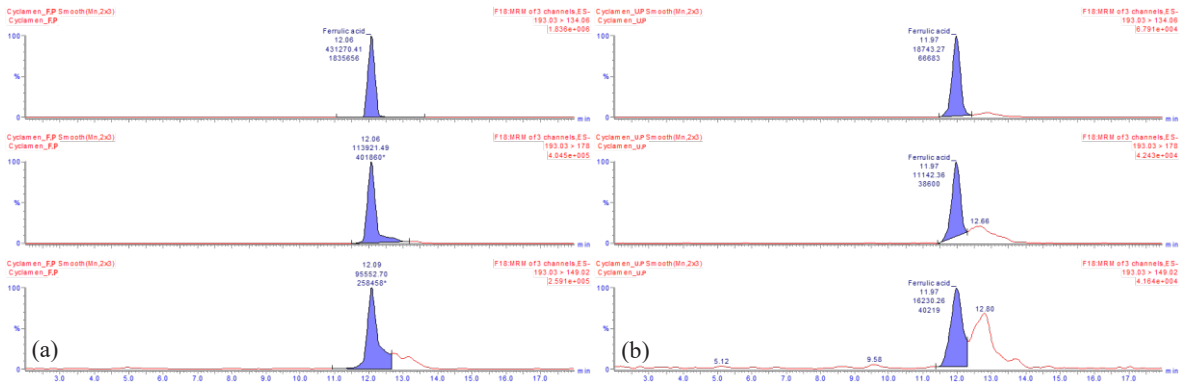


Table 2. Larvicidal activity of fresh (a) and underground (b) parts of *C. cilicium* against *M. domestica* (% ± Standard Error)

	Fresh Part	Tuber Part
Negative Control*	00.00 ± 0.00 a^y	00.00 ± 0.00 a
5 mg/mL	33.33 ± 4.81 b	22.22 ± 5.56 b
Positive Control**	100.00 ± 0.00 c	100.00 ± 0.00 c

a^y If the lower cases in the column are the same, there is no statistical difference in Duncan's multiple range test ($p > 0.05$). *Negative control: Distilled water. **Positive Control: Difluban 48 % SC.

In this assay, the larvicidal activity of *C. cilicium* against *M. domestica* was investigated and the results are shown in Table 2. In the assay, water extract was used against 2nd and 3rd instar larvae. The best result was positive control (Difluban 48 % SC, active ingredient: Diflubenzuron, CAS No: 35367-38-5), and 100 % result was observed. It was found to be effective in the fresh part of the *C. cilicium* with a value of 33.33 ± 4.81% in terms of larvicidal activity value against *M. domestica* larvae at a concentration of 5 mg / mL. Based on the results, it was seen that there was no high larvicidal activity against house fly larvae. Higher concentrations have not been tested as they could not be used effectively in the pharmacology or pesticide industry.

Table 3. Average mortality rates (%) and statistical values (mg/mL) of fresh part concentrations of *C. cilicium* against *Cx. pipiens* during the specified duration of action.

	Fresh Part 24 h later	Fresh Part 48 h later	Fresh Part 72 h later
Negative Control*	00.00 ± 0.00 a^x, A^y	00.00 ± 0.00 a, A	00.00 ± 0.00 a, A
0.1 mg/mL	0.00 ± 0.00 a, A	8.33 ± 0.00 a, B	11.11 ± 2.78 b, B
0.25 mg/mL	5.56 ± 2.78 a, A	8.33 ± 0.00 a, AB	13.89 ± 2.78 b, B
0.5 mg/mL	16.67 ± 4.81 b, A	50.00 ± 9.62 b, B	66.67 ± 4.81 c, B
1 mg/mL	36.11 ± 2.78 c, A	50.00 ± 4.81 b, B	80.56 ± 2.78 d, C
Positive Control**	100.00 ± 0.00 d, A	100.00 ± 0.00 c, A	100.00 ± 0.00 e, A
$LC_{50\ (min)}$ (mg/mL)	1.09	0.15	0.17
LC_{50} (mg/mL)	1.40 ± 0.07	0.83 ± 0.17	0.43 ± 0.09
$LC_{50\ (max)}$ (mg/mL)	2.11	4.46	1.11
$LC_{90\ (min)}$ (mg/mL)	3.05	0.07	0.22
LC_{90} (mg/mL)	5.07 ± 0.16	4.69 ± 0.42	1.48 ± 0.19
$LC_{90\ (max)}$ (mg/mL)	12.66	316.60	9.92

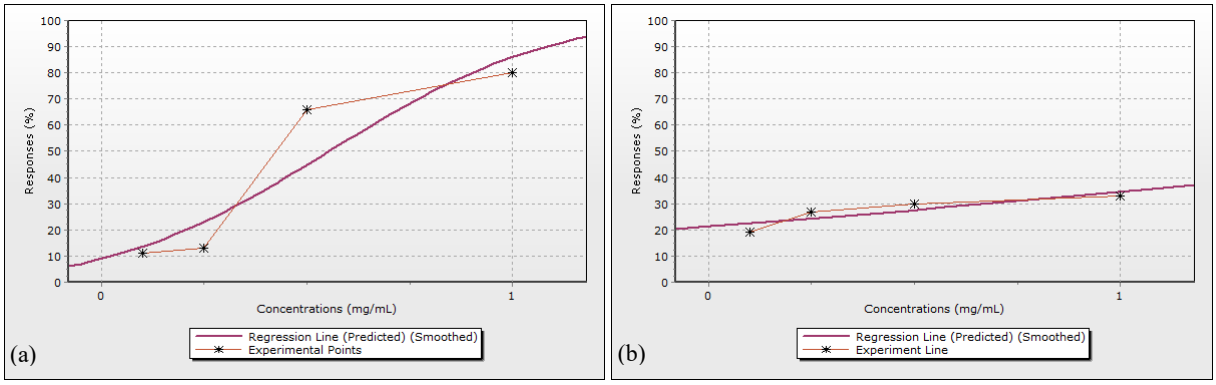
a^x : If the lower cases in the column are the same, there is no statistical difference in Duncan's multiple range test ($p > 0.05$). A^y : If the lower cases in the line are the same, there is no statistical difference in Duncan's multiple range test ($p > 0.05$). * Negative control: Distilled water. **Positive Control: Mozkill 120 SC.

Table 4. Average mortality rates (%) and statistical values (mg/mL) of tuber part concentrations of *C. cilicium* against *Cx. pipiens* during the specified duration of action.

	Tuber Part 24 h later	Tuber Part 48 h later	Tuber Part 72 h later
Negative Control	00.00 ± 0.00 a^x, A^y	00.00 ± 0.00 a, A	00.00 ± 0.00 a, A
0.1 mg/mL	2.78 ± 2.78 a, A	8.33 ± 0.00 b, A	19.44 ± 2.78 b, B
0.25 mg/mL	11.11 ± 2.78 b, A	11.11 ± 2.78 b, A	27.78 ± 2.78 bc, B
0.5 mg/mL	16.67 ± 0.00 b, A	19.44 ± 2.78 c, AB	30.56 ± 5.56 c, B
1 mg/mL	27.78 ± 2.78 c, A	38.89 ± 2.78 d, A	33.33 ± 4.81 c, A
Positive Control**	100.00 ± 0.00 d, A	100.00 ± 0.00 e, A	100.00 ± 0.00, d, A
$LC_{50\ (min)}$ (mg/mL)	1.65	1.33	1.89
LC_{50} (mg/mL)	2.90 ± 0.20	2.23 ± 0.17	9.22 ± 2.20
$LC_{50\ (max)}$ (mg/mL)	9.64	6.31	>10000
$LC_{90\ (min)}$ (mg/mL)	8.99	8.81	85.84
LC_{90} (mg/mL)	29.14 ± 0.42	28.13 ± 0.41	9513.32 ± 6.83
$LC_{90\ (max)}$ (mg/mL)	401.91	345.51	>10000

a^x : If the upper cases in the line are the same, there is no statistical difference in Duncan's multiple range test ($p > 0.05$). A^y : If the lower cases in the line are the same, there is no statistical difference in Duncan's multiple range test ($p > 0.05$). * Negative control: Distilled water ** Positive Control: Mozkill 120 SC

Figure 2. 72-hour percentage mortality graph of larvicidal activity of leaf part (a) and tuber part (b) of *C. cilicium* against *Cx. pipiens* larvae.



Larvicidal activity of *C. cilicium* extract against second and third instar larvae of *Cx. pipiens* are shown in Table 3 and Table 4. Water extracts of *C. cilicium* were used for larvicidal activity against *Cx. pipiens*. The best result was positive control (Mozkill 120 SC, active ingredient: Spinosad, CAS No: 168316-95-8), and 100 % result was observed within 1 hour. After 72 hours of exposure, the fresh part showed the most toxic effect, with 0.43 ± 0.09 mg/mL, LC_{50} results. Concentration and time of exposure were found to be effective in increasing larvicidal activity (Figure 2). In the larvicidal study of *Cyclamen alpinum* Dammann ex. Springer extracts against *Cx. pipiens*, it was found that the leaf part was more lethal, with a value of 90 ± 1.33% (0.534 mg / mL, LC_{50}) at 1 mg / mL. (Turan and Mammadov, 2018). The larvicidal activity of *Cyclamen mirabile* Hildebr. and *C. alpinum* tuber extracts against *Cx. pipiens* was investigated. Tuber extract was applied at different concentrations (100-1000 ppm) in the larval stages. The extracts' LC_{50} values were determined according to the larvae's mortality rates at different periods (12th-24th-48th-72nd-96th). According to the results, when the LC_{50} values were compared, it was observed that *C. mirabile* species (86.2 ppm) was more active than *C. alpinum* species (161.3ppm) (Oz *et al*., 2013). The results of the study are compatible with the literature and it has been found that it shows a good larvicidal activity after 72 hours.

4. CONCLUSION

There are no studies on larvicidal and toxic effects in the literature of *C. cilicium*. This research reveals that this species, especially the leaf part, has a strong biological activity and shows activity in very small concentrations. However, no larvicidal power was observed against *M. domestica*. Therefore, these results suggest that the *C. cilicium* leaf part could be a potential candidate for new potential biocide methods and the development of excellent sources of antioxidant molecules.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Murat Turan: Investigation, Formal analysis, Writing-original draft, Writing-review & editing. **Ramazan Mammadov:** Funding acquisition, Investigation, Writing-review & editing.

Orcid
Murat Turan https://orcid.org/0000-0003-2900-1755
Ramazan Mammadov https://orcid.org/0000-0003-2218-5336

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Oleandrin Activates Apoptosis and Inhibits Metastasis of A375 Human Melanoma Cells

Canan Eroglu Gunes[1*], Fatma Secer Celik[1], Mucahit Secme[2], Ercan Kurar[1]

[1] Department of Medical Biology, Meram Faculty of Medicine, Necmettin Erbakan University, Konya, Turkey
[2] Department of Medical Biology, Faculty of Medicine, Pamukkale University, Denizli, Turkey

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Abstract

Skin cancer has an increasing incidence worldwide. Non-melanoma skin cancers and malignant melanomas are the most common skin malignancy. *Nerium oleander* L., which is a member Apocynaceae family, has historically been used in the treatment of hemorrhoids, leprosy and snake bites. Oleandrin is one of the cardiac glycosides obtained from *N. oleander*. The study aimed to evaluate the anticancer effects of oleandrin in A375 human melanoma cells via apoptosis, metastasis and invasion pathways. The effect of oleandrin on cell viability was evaluated using an XTT cell viability assay. Expressions of 8 genes in apoptosis and 10 genes in metastasis and invasion were determined by RT-qPCR. The IC_{50} dose of oleandrin was found to be 47 nM for 48 h in A375 melanoma cells using CompuSyn version 1.0 software. Oleandrin treatment significantly increased the expression of *CASP9, FAS, CYCS, CDH1* and *TIMP3*; however, significantly decreased the expression of *BCL2, P53, CDH2* and *TGFB3* in A375 cells. In conclusion, changes in expression levels of apoptosis, metastasis and invasion genes indicated that oleandrin has an apoptotic and anti-metastatic effect in A375 cells.

Corresponding Author:
Canan Eroglu Gunes, Department of Medical Biology, Meram Faculty of Medicine, Necmettin Erbakan University, Konya, Turkey, cananeroglu88@gmail.com
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1. INTRODUCTION

Cancer is an important public health problem all over the world (Siegel *et al*., 2020). The incidence of skin cancer has been gradually increasing. It is reported that the incidence of skin cancer is one in 33 people in men and 52 people in women. The most common skin malignancies in the Caucasian race are reported as non-melanoma skin cancers and malignant melanomas (American Cancer Society, 2016). It is known that the incidence of melanoma has rapidly also increased in the last 30 years. Although invasive melanoma accounts for approximately 1% of all skin cancer cases, it constitutes the majority of deaths due to skin cancer (American Cancer Society, 2020). Alone surgical resection of early-stage melanoma often resulted in recovery, however, survival rate significantly decreases in later-stage melanoma (Gershenwald *et al*., 2017). Although chemotherapies, which are quite common in cancer treatments, have been used for a long time, they inevitably have side effects. Therefore, researchers are looking for alternative agents that target cancer cells and have minimal or no effect on normal cells.

Recently, unconscious consumption of herbal products has been increased because of the therapeutic effects of these products. Although some plant extracts turn into medicines in time, they are also directly consumed. One of these plants is *Nerium oleander*, which is known to be toxic, and it is consumed because it contains cardiac glycosides. *N. oleander*, a member of the Apocynaceae family, is a tropical flowering plant with white, red and pink flowers (*Carbik et*

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al., 1990). This plant is commonly known as oleander. Although widely cultivated in Southwest Asia, no precise region of origin has been identified (Li *et al*., 1964). *N. oleander* is widely used as an ornamental plant in paysage, parks and roadsides due to its showy and fragrant flowers, and grows well in hot subtropical regions (Kumar *et al*., 2013). *N. oleander* is one of the most poisonous of commonly grown garden plants (Li *et al*., 1964). The unconscious use of these poisonous plants can lead to very serious consequences. For this reason, there is an increasing interest in *N. oleander* extracts and their some active ingredients in the literature to reveal the mechanism of effect. *N. oleander* has historically been used in the treatment of hemorrhoids, leprosy, and snakebite (Szabuniewicz *et al*., 1972). It has also been reported that the active ingredients of *N. oleander* have various biological activities such as cardiotonic (Kolkhof *et al*., 2010), diuretic (Deng, 1959), antibacterial (Huq *et al*., 1999) and antitumor (Shiratori, 1967; Hartwell & Abbott, 1969).

Cardiac glycosides are secondary compounds found in amphibians and plants. Its mechanisms are based on the inhibition of sodium-potassium (Na^+/K^+)-ATPase by providing a positive inotropic effect. Oleandrin is one of the cardiac glycosides obtained from *N. oleander* used in the treatment of cardiac abnormalities (Hung, 1999). Some well-known cardiac glycosides are ouabain, bufalin, digoxin and digitoxin. These have similar chemical structures as they contain sugar, steroid and lactone ring (Botelho *et al*., 2019). The therapeutic uses of cardiac glycosides are limited by their cardiovascular toxicity (Schoner & Scheiner-Bobis, 2007; Newman *et al*., 2008). To overcome this problem, cardiac glycoside-derived compounds should be investigated for the capacity of inhibiting the proliferation and metastasis in cancer cells without causing cardiovascular toxicity. It was shown that cardiac glycosides in nanomolar concentrations are not toxic for normal cells and it has an antiapoptotic and proliferative effect on them, while these drugs inhibit proliferation of cells and induce apoptosis in cancer cells (McConkey *et al*., 2000; Trevisi *et al*., 2004).

Inhibitors of Na^+/K^+-ATPase pump have an interest as candidates for cancer therapy (Pressley, 1996). The abnormal expression and activity of this pump have been reported to play role in the progression of various aggressive cancer types (Blok *et al*., 1999; Rajasekaran *et al*., 1999). Cardiac glycosides have been used to treat congestive heart disease. Because those selectively inhibit Na^+/K^+-ATPase and cause increases of intracellular Ca^{2+}concentration (Mans *et al*., 2000).

Oleandrin, a type of polyphenolic cardiac glycosides isolated from the leaves of *N. oleander*, was used in the treatment congestive heart failure due to the inhibition pump of Na^+/K^+-ATPase (Patel, 2016). Cardiac glycosides bind to the Na^+ pump in failed cardiac muscle fibers. Therefore, this situation causes an increase in intracellular Na^+ as the pump's activity inhibits. The increase of intracellular Na^+ causes a simultaneous increasing of cytosolic Ca^{2+} and provides increasing of contractile force (Rose & Valdes, 1994). Inhibitors of Na^+/K^+-ATPase can cause the death of cancer cells through the production of reactive oxygen species, apoptosis, autophagy or cell cycle arrest (Durlacher *et al*., 2015).

It has been shown that oleandrin (1.7-170 nM) induced apoptosis in many cancer cells such as PC-3 (McConkey *et al*., 2000; Smith *et al*., 2001), DU145 (Smith *et al*., 2001), U937 (Watabe *et al*., 1996; Watabe *et al*., 1997; Manna *et al*., 2000), HeLa, CaCOV3 and Jurkat (Manna *et al*., 2000). Although the anticarcinogenic activity of oleandrin in many cancer cells has been shown in previous studies, the mechanism underlying this effect has not been fully elucidated. Therefore, it was aimed to determine the effect of oleandrin on apoptosis, metastasis and invasion pathway genes in melanoma cells. For this purpose, the cytotoxic effect of oleandrin was evaluated in A375 human melanoma cells. The anti-carcinogenic effect of oleandrin was determined by evaluating expression changes of some genes role in cancer steps including apoptosis, metastasis and invasion.

2. MATERIAL and METHODS

2.1. Chemicals

Commercial Oleandrin (99% HPLC) was obtained from Sigma (USA). DMEM, PBS, penicillin/streptomycin, XTT kit and FBS were obtained from Biological Industries. QIAzol was obtained from Qiagen, USA. cDNA synthesis kit was obtained from Bio-Rad.

2.2. Cell Culture

A375 human melanoma cell line (ATCC®CRL-1619™) was purchased from ATCC. A375 cells were proliferated in DMEM medium containing 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin in an incubator containing humidity with 5% CO_2 atmosphere and 37 ^oC.

2.3. Cytotoxicity Assay

Cytotoxic activity of oleandrin was detected in A375 cells by using XTT colorimetric based assay. And then the IC_{50} dose was calculated. Oleandrin was dissolved in methanol. A375 cells were seeded as $1x10^3$ cells/well in a 96-well plate. The cells were treated with various doses of oleandrin (7.5, 15, 30, 40, 50, 100, 150 and 300 nM) for 24, 48 and 72 hours. Then, amounts of cell viabilities were determined at wavelength 450 nm (reference wavelength 630 nM) using a microplate reader.

"Viability (%) = Absorbance value of experiment well / Absorbance value of control well x 100" formula was used for cell viability calculation.

2.4. RNA Isolation, cDNA Synthesis and RT-qPCR Analysis

Total RNA isolation from A375 cells and cDNA synthesis were conducted with QIAzol (Qiagen) and Transcriptor first-strand cDNA synthesis kit (Bio-Rad) using manufacturer's manuals, respectively. The primer sequences of genes used in this study for RT-qPCR analysis were designed with IDT PrimerQuest (https://eu.idtdna.com/site) and were presented in Table 1. The RT-qPCR reaction mix was prepared for each gene. Briefly, each reaction contained 2 µl cDNA, 5 pmol of forward and reverse primer and 5 µl 2X Sybr Green Supermix (Bio-Rad). RT-qPCR protocol was performed as initial denaturation at 95°C for 10 minutes, denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds, respectively. PCR reaction was performed as 40 cycles. RT-qPCR analysis was evaluated using Bio-Rad CFX Connect™ Real-Time System. Analysis of melting curves was performed by progressive heating from 65 °C to 95 °C on the PCR products. ACTB gene was used as a reference gene in the study.

2.5. Statistical Analysis

All experimental data were repeated three times with data shown as mean±SD (standard deviation). The RT-qPCR analysis was performed via the $2^{(-\Delta\Delta Ct)}$ method. The comparisons between groups have been assessed in RT^{2}Profiles™PCR Array Data Analysis, which is evaluated statistically using the ''Student *t*-test''.

3. RESULTS and DISCUSSION

The effects of oleandrin on cell viability were investigated with XTT method in A375 cells. Oleandrin inhibited cell proliferation in A375 human melanoma cells (Figure 1). The IC_{50} dose of oleandrin was found to be 47 nM for 48 h in A375 cells using CompuSyn version 1.0 software. In the following experiments, the dose of 47 nM was used in A375 cells.

In the literature, the effects of cardiac glycosides in various types of cancer have been investigated such as breast cancer (Chen *et al*., 2006), non-small cell lung cancer cells (Frese *et al*., 2006), prostate cancer cells (Yeh *et al*., 2001) and leukemia cells (Turan *et al*., 2006).

Oleandrin also has structural similarities with other glycosides. Cardiac glycosides have almost the same properties as oleandrin (Kumar *et al*., 2013). Oleandrin obtained from *N. oleander* leaves has been shown to have tumor cell growth inhibitory and anti-inflammatory effects (Hung, 1999; Stenkvist, 1999). However, the mechanism underlying this effect has not been fully elucidated. In a study evaluating the effect of oleandrin, one of the cardiac glycosides, on colon cancer, it was found that the IC_{50} value for SW480 colon cancer cells was 0.02 µM and 0.56 µM for NCM460 normal colon cells (Pan *et al*., 2017).

Table 1. Primers sequences of genes used in this study for RT-qPCR analysis.

Gene name	Primer sequence	PCR product size (bp)
BAX	F:5- GGAGCTGCAGAGGATGATTG-3 R:5- GGCCTTGAGCACCAGTTT-3	151
BCL2	F:5- GTGGATGACTGAGTACCTGAAC-3 R:5- GAGACAGCCAGGAGAAATCAA-3	125
CASP3	F:5-GAGCCATGGTGAAGAAGGAATA-3 R:5-TCAATGCCACAGTCCAGTTC-3	162
CASP7	F:5-CGAAACGGAACAGACAAAGATG-3 R:5-TTAAGAGGATGCAGGCGAAG-3	169
CASP9	F:5-CGACCTGACTGCCAAGAAA-3 R:5-CATCCATCTGTGCCGTAGAC-3	153
CYCS	F:5- GGAGAGGATACACTGATGGAGTA-3 R:5- GTCTGCCCTTTCTTCCTTCTT-3	102
FAS	F:5- GTGATGAAGGACATGGCTTAGA-3 R:5- GCCCAAACTTCACAGCATTAG-3	156
P53	F:5- GAGATGTTCCGAGAGCTGAATG -3 R:5- TTTATGGCGGGAGGTAGACT -3	129
CDH1	F:5-GAGAGCGGTGGTCAAAGAG-3 R:5-AGCTGGCTCAAGTCAAAGT-3	117
CDH2	F:5-GCTGACCAGCCTCCAAC-3 R:5-CATGTGCCCTCAAATGAAACC-3	112
COL4A2	F:5-AAGTACAGCTTCTGGCTGAC-3 R:5-AGCGGCTGATGTGTGTG-3	103
CD44	F:5-TTCTGGATCAGGCATTGATGA-3 R:5-GTCCAGTCCTGGTTCTGTTT-3	99
TGFB1	F:5-GCCTGGACACGCAGTACA-3 R:5-TTGCAGGAGCGCACGAT-3	172
TGFB2	F:5-AAAGACAGGAACCTGGGATTTA-3 R:5-TGTGGAGGTGCCATCAATAC-3	135
TGFB3	F:5-CTTGCAAAGGGCTCTGGT -3 R:5-CTAATGGCTTCCACCCTCTTC-3	120
TIMP1	F:5-GCGTTATGAGATCAAGATGACCA-3 R:5-AACTCCTCGCTGCGGTT-3	141
TIMP2	F:5-GCTGCGAGTGCAAGATCA-3 R:5-CTCTTGATGCAGGCGAAGAA-3	136
TIMP3	F:5-GCAAGATCAAGTCCTGCTACTAC-3 R:5-GGATGCAGGCGTAGTGTTT-3	123
ACTB	F:5-TGGCTGGGGTGTTGAAGGTCT-3 R:5-AGCACGGCATCGTCACCAACT-3	179

Following the total RNA isolation, cDNA synthesis was performed. Expressions of *BAX*, *BCL2*, *CASP3*, *CASP7*, *CASP9*, *CYCS*, *FAS* and *P53* genes for apoptosis and, *CDH1*, *CDH2*, *COL4A2*, *CD44*, *TGFB1*, *TGFB2*, *TGFB3*, *TIMP1*, *TIMP2* and *TIMP3* genes for metastasis and invasion were determined by RT-qPCR. It was defined that oleandrin in A375 cells caused a significant increase in the expression of *CASP9*, *CYCS* and *FAS*, and a significant decrease in the expression of *BCL2* and *P53* genes that play an important role in apoptosis. On the other hand, in A375 cells, a significant increase was seen in the expressions of *CDH1* and *TIMP3* genes and a decrease was seen in the expressions of *CDH2* and *TGFB3* genes, which play an

important role in metastasis and invasion, in the dose group, compared with the control group (Figure 2, $p < 0.05$).

Figure 1. Effect of oleandrin on the viability in the A375 cells. IC_{50} dose of oleandrin in A375 cell line was found to be 47 nM.

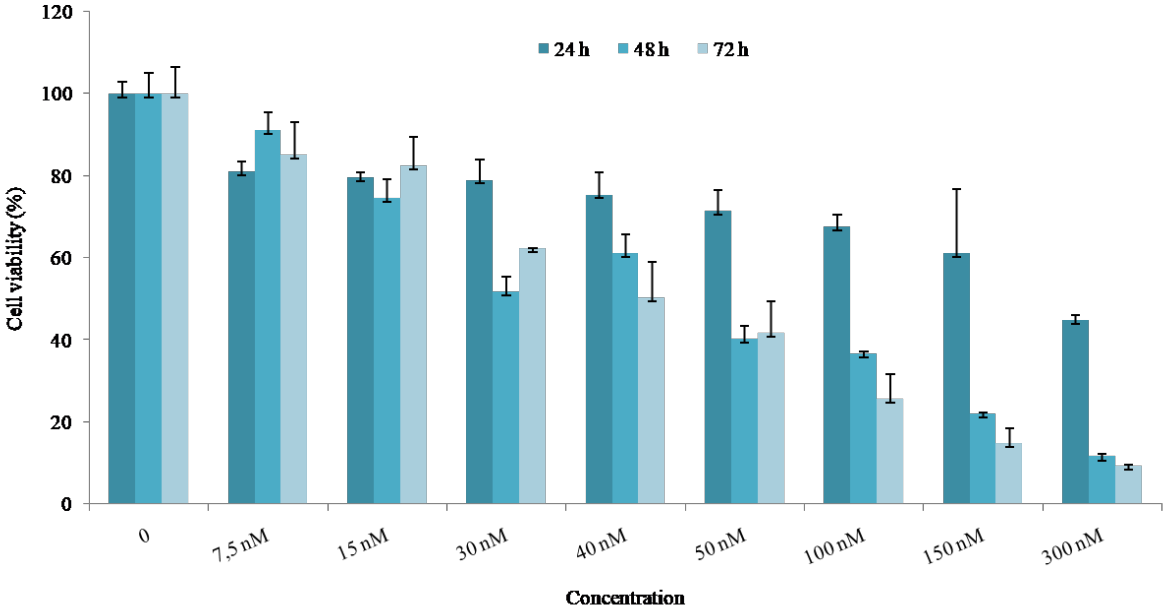
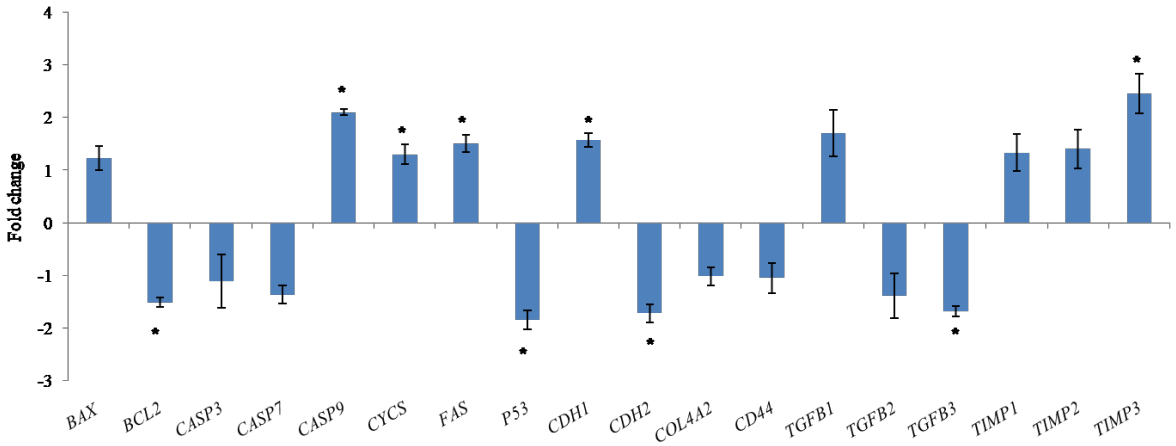


Figure 2. The expression changes of apoptosis, metastasis and invasion related genes in oleandrin treated A375 cells. * indicates statistically important ($p < 0.05$).



Recently, it was reported that cardiac glycosides play roles in the development of cancer and metastasis via the regulation of different cellular pathways (Lin *et al*., 2010). It is also known that some cardiac glycosides such as digitoxin and digoxin inhibit cancer progression via inducing the immunogenic death of cancer cells (Kepp *et al*., 2012). In another previous study, Anvirzel, which is mainly composed of oleandrin and oleandrigenin cardiac glycosides, has been shown to regulate cell death via inhibiting GSK-3 and NOS activity in U87 human glioma cells (Terzioglu-Usak *et al*., 2020).

Oleandrin has been reported to induce apoptosis, not necrosis, by increasing Fas expression in many tumor cells (Jurkat, HL-60, HuT-78, HeLa, SKOv3, MCF-7 and U-937). However, it has been reported that oleandrin does not significantly alter the rate of apoptosis in peripheral blood mononuclear cells. It has also been reported that FADD negative tumor cells inhibit oleandrin-induced apoptosis (Sreenivasan *et al*., 2006). In a study investigating the effect of oleandrin on human prostate cancer, it has been shown that oleandrin caused apoptosis in human prostate cancer cells and also stimulated intracellular calcium increase (McConkey *et al*., 2000). In addition, it has been reported in another study that oleandrin induces apoptosis via modulating intrinsic apoptotic pathway and ER stress in breast cancer (Li *et al*., 2020). Another study showed that oleandrin suppressed cell proliferation, colony formation and invasion through inhibition of the STAT-3 pathway in breast cancer cells (Ko *et al*., 2018). In another study, oleandrin was reported to induce mitochondria-mediated apoptosis in human colon cancer cells (Pan *et al*., 2017). Newman *et al*. (2007) showed that oleandrin stimulated autophagy by increasing LC3-II expression in PANC-1 human pancreatic cancer cells. It was concluded that oleandrin inhibited FGF-2 expression in PC3 and DU145 prostate cancer cells in a time and dose-dependent manner (Smith *et al*., 2001). Results of a previous study showed that oleandrin increased the sensitivity to radiation of PC-3 human prostate cells. In addition, the sensitivity to oleandrin and radiation-induced apoptosis of PC-3 cells has been shown to be associated with caspase-3 activation (Nasu *et al*., 2002). Oleandrin administration in CD-1 mice before the administration of skin tumor promoter TPA (l2-O-tetradecanoylphorbol-13-acetate) caused a significant decrease in epidermal COX-2 (cyclooxygenase-2) activity and ODC (ornithine decarboxylase) expression, which are important in skin edema, hyperplasia, inflammation and tumor progression. In addition, oleandrin treatment before TPA administration caused a decrease in TPA-induced PI3K, Akt and NF-κB expression (Afaq *et al*., 2004). In a previous study, it has been reported that oleandrin and cisplatin have a synergistic effect in human osteosarcoma cells via regulating apoptosis and the p38 MAPK pathway (Yong *et al*., 2018). In addition, a study conducted by the same research group indicated that oleandrin did not significantly affect the viability of normal hFOB1.19 human osteoblast cells when treated with various concentrations of oleandrin (0, 25, 50, 75, 100 and 150 nM). However, oleandrin has been shown to cause an increase of ROS and a decrease of mitochondrial membrane potential (MMP) in osteosarcoma cells (Ma *et al*., 2016). In another study, Ma *et al*. (2015) showed that oleandrin suppressed the Wnt/beta-catenin signalling pathway in U2OS and SaOS-2 osteosarcoma cells. It has also been reported that oleandrin suppressed the invasion of osteosarcoma cells (Ma *et al*., 2015). A previous study illustrated that oleandrin induced apoptosis in SW480 and RKO colon cancer cells. It was reported that oleandrin showed this apoptotic effect by causing an increase in caspase-3, caspase-9, cytochrome c, BAX expression and a decrease in Bcl-2 expression (Pan *et al*., 2017). In a previous study, oleandrin (2.5-160 ng/ml) and Apo2L/TRAIL treatment alone did not cause apoptosis in Calu1 lung cancer cells, which are highly resistant to Apo2L / TRAIL, but the combined treatment in cells resulted in apoptosis by sensitizing it apoptosis induced by Apo2L/TRAIL. However, it has been reported that oleandrin at the same doses was not susceptible to Apo2L/TRAIL-induced apoptosis in normal PBMCs (Frese *et al*., 2006). In a previous study, a model was created with human or mouse glioma cells in mice and the effect of oleandrin treatment on tumor progression was evaluated. In both models, oleandrin reduced tumor size and inhibited cell proliferation. It was shown that oleandrin could not increase brain-derived neurotrophic factor (BDNF) expression and decrease tumor volume in mice in which BDNF was suppressed. It was thereby emphasized that BDNF is important in the protective and antitumor effect of oleandrin. It has also been reported that oleandrin can change the tumor microenvironment by increasing the level of BDNF in the brain parenchyma (Garofalo *et al*., 2017). In another previous study, it has been shown that oleandrin has a BDNF-mediated

neuroprotective effect (Van Kanegan *et al*., 2014). Oleandrin has also been shown to provide an important protective effect to injured neural tissues due to glucose and oxygen deprivation (Dunn et al., 2011). The neuroprotective activity of oleandrin has been attributed in part to the channel arrest and metabolic strategy against hypoxia (Piccioni *et al*., 2004).

4. CONCLUSION

In this study, anticancer effects of oleandrin were investigated in A375 human melanoma cells. It exhibited an apoptotic effect in human melanoma cells by changes in expression levels of apoptosis genes. Moreover, it caused anti-metastatic and anti-invasive effects by changes in expression levels of genes related to metastasis and invasion. Therefore, it is thought that oleandrin can be used as an anti-cancer agent exhibiting anti-proliferative, apoptotic, anti-metastatic and anti-invasive effects in human melanoma cells. However, further *in vitro* and *in vivo* research related to the anticancer activity of oleandrin should be performed to elucidate molecular biological mechanisms in cellular processes such as apoptosis and metastasis in human melanoma.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Canan Eroglu Gunes: Investigation, Writing, Methodology. **Fatma Secer Celik:** Methodology, Writing, Review, Editing. **Mucahit Secme:** Writing, Review, Editing, Visualization. **Ercan Kurar:** Writing, Review, Editing.

Orcid

Canan Eroglu Gunes https://orcid.org/0000-0002-3796-575X
Fatma Secer Celik https://orcid.org/0000-0001-5619-8958
Mucahit Secme https://orcid.org/0000-0002-2084-760X
Ercan Kurar https://orcid.org/0000-0002-9234-1560

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The Effect of Various Impregnating Agents on the Adhesion Strength of Solution Concentration Structure

Hatice Ulusoy[1*], Huseyin Peker[2]

[1] Department of Forestry, Köyceğiz Vocational School, Muğla Sıtkı Koçman University, Muğla, Turkey
[2] Department of Forest Industrıal Engineering, Forest Faculty, Artvin Çoruh University, Artvin, Turkey

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Abstract

Water-based solvents and preservatives have been increasingly significant in terms of human and environmental health in this century. The effect of impregnated vacuum and diffusion time on the adhesion resistance of Spruce (*Picea orientalis* (L.) Link.) wood impregnated with Timbercare Aqua content and adhered with polyurethane glue was investigated in this research, and thus the areas of use in the wood industry were tried to be determined. The cuts were made according to the standards specified in the test sample preparation. All samples have been checked for fibrousness, smoothness, cracks, knots, and color flaws (fungus, rot, etc.). In determining the adhesion level, 100 x 50 x 30 mm test specimens were prepared and in determining the adhesion resistance change, it was carried out according to TS EN 205 standard (150 x 20 x 0.75 mm). According to the results of the experiments, the highest percent retention was achieved at 30 minutes vacuum and 40 minutes diffusion (2.78%), and the lowest was achieved at 30 minutes vacuum 50 minutes diffusion (1.96 %). The highest air dry specific gravity for 30 minutes vacuum and 50 minutes diffusion as 0.43 g/cm^3), highest full dry specific gravity for 30 minutes vacuum and 50 minutes diffusion as 0.41 g/cm^3), highest adhesion resistance for 30 minutes vacuum and 40 minutes diffusion as 2.70 N/mm^2, at lowest adhesion resistance value for 30 minutes vacuum and 60 minutes diffusion as 2.61 N/mm^2) was determined.

Corresponding Author:
Hatice Ulusoy, Department of Forestry, Köyceğiz Vocational School, Muğla Sıtkı Koçman University, Muğla, Turkey, hatice.tirasulusoy@gmail.com
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1. INTRODUCTION

The natural production of different materials by gluing wood was performed by the Egyptians at least 3,000 years ago, and the concept of gluing wood with some glue dates back to the beginning of human history (Keimel, 2003). It is very significant for the impregnation materials applied to the wood material to provide protective properties as well as to be compatible with the adhesive material for adhesion. It is known that impregnating wood with pressure decreases glue adhesion resistance. It has been stated that the type of impregnation, the amount of impregnation retention, the interaction of the preservative with the surface greatly affect the adhesion resistance of the wood material in glued joints (Vick & Christiansen, 1993). The necessity of preserving the wood material in the usage was accepted even centuries ago, and it is seen that various measures were taken. Archaeological excavations and examination of sunken ships showed that protection of wood content with partially charred emerged as the first measure taken 4000 years ago. The "Diana Temple" in Ephesus, as an example, is supported by charred wooden poles. The application of adhesives goes back to ancient Egyptian times. In the seventeenth century, wood glue was first used commercially (Huş, 1977). Wood material, which is the primary raw material used in the woodworking industry, is one of the natural resources that is currently depleting. However, in case of proper use and protection, it

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can meet the increasing need for wood raw material. The wood raw material is used in a wide variety of areas by transforming it into massive or various plate and composite products, and its texture in different parts distinguishes it from other decorative materials. Since the wood material is hygroscopic, it exchanges moisture with the surrounding air to reach a balanced humidity suitable for the temperature and relative humidity of the air in the place of use, if this exchange occurs under the fiber saturation point, it changes its size and volume, it can burn, it can be destroyed by biotic and abiotic factors. (Keskin *et al*., 2008). The impregnation procedure involves penetrating various chemical compounds into the wood in special facilities in accordance with specific requirements and norms to avoid the decay, burning, and dimensional changes in the wood. The wood material's life is extended by 7-8 times as a result of the impregnation procedure (Özçifçi & Batan, 2009). Impregnated wood materials are resistant to fungi and insects, easy to set up, lower maintenance and repair costs compared to other alternative products and economic, and thus, wood materials are impregnated with various wood preservative chemicals (Dizman, 2005). In a study, experimental samples prepared from scotch pine (*Pinus sylvestris* L.) and eastern beech (*Fagus orientalis* Lipsky.) wood were prepared by % of polyvinyl acetate (PVAc), Urea-formaldehyde (UF), Melamine-formaldehyde (MF) and Phenolformaldehyde (FF) adhesives among themselves prepared with modifications of 0,15,35, 50. According to TS EN 205 (2004) guidelines, the research samples were stored in three different conditions. The test samples were then put through a tensile test in accordance with DIN 53 255 principles. At the conclusion of the tests, it was discovered that the glue modification had no impact on adhesion performance in standard atmosphere, but improved adhesion performance in a cold water keeping and boiling environment (Altınok *et al*., 2000). Oriental beech, sessile oak and scotch pine woods bonded with Klebit 303, Kleiberit 305.0 and Super Lackleim 308 adhesives; The highest parallel bonding strength to fibers was obtained in Eastern beech and sessile oak using Klebit 303 glue, the lowest in sessile oak using Super Lackleim 308 glue and yellow pine using Klebit 303 glue (Özçifçi *et al*., 1999). In research, the adhesion resistance of Eastern beech (*Fagus orientalis*), sessile oak (*Quercus petraea* spp.) and Scotch pine (*Pinus sylvestris*) wood treated with Klebit 303, Kleiberit 305.0 and Super Lackleim 308 adhesives were determined (Örs *et al*., 2000). According to the results of the study, the highest adhesion strength was obtained with Klebit 303 glue, Eastern beech (89,80 N/mm²) and sessile oak (89,50 N/mm²), the lowest adhesion strength with Super Lackleim 308 glue and Sapless oak (50,18). N/mm²) and Scotch pine (50,45 N/mm²) with Klebit 303 glue. Adhesion strength values were determined by impregnating some wood materials. The highest value of Klebit glue 303 (11.84 N/mm²) in Eastern beech impregnated with the dipping method and Tanalith-CBC, the lowest value with kleberite 305.0 glue (3.1 N/mm²) in scotch pine wood impregnated with vacuum method and Tanalith-CBC was determined (Örs *et al*., 2000). Adhesion resistance to the surface depending on the type of wood was determined as the highest Eastern beech (7.347 N/mm²) and the lowest scotch pine (5.087 N/mm²). They reported that the adhesion resistance in eastern beech was 13% more than sessile oak and 44% more than yellow pine, and they stated that the wood type was effective in the adhesion resistance and this effect may be due to the main density and surface properties (Atar & Keskin, 2004). Since wood is applied anywhere in the ecological world (human/environment), so the protection of the material can only be achieved by the use of organic or organic materials. In this context, the impregnation process was carried out with *Timbercare Aqua*, one of the water-based wood preservative materials, and the effect of polyvinyl acetate glue on the adhesion resistance of the material Spruce wood (*Picea orientalis* (L.) Link.) was investigated by keeping the vacuum and diffusion times different, thus the areas of use and effect in the wood industry were attempted to be determined.

2. MATERIAL and METHODS

2.1. Material

Spruce wood (*Picea orientalis* (L.) Link.), one of the wood species of our country, was chosen in the research and the sample cuts were obtained from the sapwood sections in the radial direction accoriding to the standards. The impregnation material was applied as Timbercare Aqua, and the glue was polyvinyl acetate (PVA) type TS 53 (1981), TS 2470 (1976).

2.2. Method

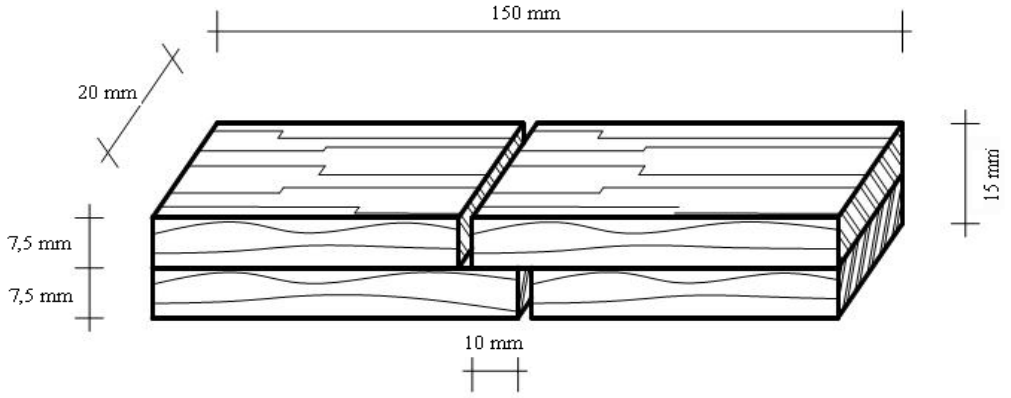
2.2.1. Experiment Sample Preparation

The cuts were prepared according to the standards specified in the test sample preparation. All samples were checked for fibrousness, smoothness, cracks, knots, and color defects (fungus, rot, etc.). In order to determine the adhesion standard, 100 x 50 x 30 mm test specimens were prepared and it was carried out according to TS EN 205 (2004) standard (150 x 20 x 0.75 mm) in determining the change in adhesion resistance.

2.2.2. Adhesion Strength (N/mm^2)

Samples were prepared in net dimensions of 10 x 20 x 150 mm, with a part thickness of 5 mm in accordance with the principles specified in TS EN 205 (2004) standards. According to glue type, temperature, holding time, cross-section, delivery speed, recurrence, and tree type, the sample was prepared to represent each group. With the Universal test unit, it was attempted to be removed from the glue line by applying a gradual pulling force with a loading speed of 5 mm/min to the adhesion surface of the test specimens. Polyurethane glue was applied as glue and the manufacturer's criteria were followed. The maximal force at break (Fmax) is used to calculate adhesion resistance (y); A = an x b = adhesion surface area (mm^2). The adhesion strength was calculated from the following equation.

Figure 1. Adhesion Strength Test (Altınok *et al*.,2009)



2.2.3. Impregnation

The impregnation procedure was carried out in accordance with ASTM-D 1413-76 (1976). Vacuum of 30 minutes and a diffusion time of 30 minutes was applied. After the impregnation and diffusion processes, the samples were kept in an air-dry condition for a while. First measurement and weighting of all samples were carried out, then full dry weighting/measurement was performed after the oven at 103 ± 2 ºC at the end of 24 hours and then vacuum impregnation was performed.

2.2.4. Retention

After the impregnation process, the residual content (retention (%)) in proportion to the fully dry wood weight was calculated using the formula (1) (Baysal & Yalınkılıç, 2005).

$$R\ (\%) = Wfdsgai\text{-}Wfdsgbi\ /\ Wfdsgbi\ x\ 100 \quad (1)$$

Wfdsgai = Full dry specific gravity after impregnation (g)

Wfdsgbi = Full dry specific gravity before impregnation (g)

2.2.5. Air/Full Dry Specific Gravity

Specific weights were determined in line with the principles of TS 2471 (1976) and TS 2472 (1976), and all samples were kept until they reached constant weight at 20 ± 2 °C temperature and 60 ± 5 % relative humidity and then all procedures were performed.

2.3. Statistical Evaluation

In order to determine the effect of the impregnation material on the wood groups in the statistical evaluation, a Simple Analysis of Variance (SAV) was performed, and homogeneity groups were formed according to the Duncan Test (HG) results.

3. RESULTS and DISCUSSION

3.1. Solution Properties

Findings regarding solution properties are given in Table 1.

Table 1. Solution properties used in the impregnation process.

Impregnation Material (%1)	Solvent	Temperature (ºC)	Ph		Density (g/ml)	
			BI	AI	BI	AI
Timbercare Aqua	DW	22 ºC	6.86	6.86	0.990	0.990

DW: Distilled water *BI: Before Impregnate *AI: After Impregnate

There was no difference in pH and density before and after the impregnation process of "*Timbercare Aqua*", a Hemel product. This may result from the wood type, anatomical structure and preservative content.

3.2. Retention (%)

Retention (%) (net dry impregnation material amount) is presented in Table 2.

Table 2. The amount of substance that adheres to the wood after the impregnation process (%) values

Impregnation Material	Vacuum Time (Min)	Diffusion Time (Min)	Retention (%)	HG
Timbercare Aqua	30	30	2.56	B
		40	2.78	A
		50	1.96	D
		60	2.01	C

HG: Homogeneity groups

The highest % retention was at 30 minutes vacuum and 40 minutes diffusion (2.78 %) and the lowest at 30 minutes vacuum and 50 minutes diffusion (1.96 %). The effects of barite on wood impregnation and density were investigated in there search. As a result of these experiments, the total retention value was determined as a maximum 50% barite solution (20.30 kg/m^3) in beech wood (Tan & Peker, 2015). In research, the total retention and % retention in wood material impregnated with tea plants was analyzed. The lowest % retention in iroko wood (1.58 %) and the highest % retention rate in beech wood (6.75 %) were observed as a result of

experiments. They reported that the lowest total retention was in iroko (31.27 kg/m^3) and the highest total retention value was in beech wood (100.65 kg/m^3). According to the retention results; it is stated that organics obtained from tea plant extracts can be applied as preservatives in wood material (Atılgan *et al*., 2013). They determined the highest amount of retention in yellow pine (19.39 kg/m³ - 21.81%) and least in oak (8.742 kg/m³ - 9.15 %); They reported that obtaining the highest values in scotch pine samples may be due to the fact that coniferous trees have open passage pairs that provide fluid flow in the longitudinal direction and thus store excess impregnation material (Özçifçi & Batan, 2009).

3.3. Air/Full Dry Specific Gravity

Air and full dry specific gravity change is given in Table 3.

Table 3. Air/Total Dry Specific Gravity Change (g/cm^3)

Impregnation Material	Vacuum Time (Min)	Diffusion Time (Min)	Air Dry Specific Gravity (g/cm^3)	Full Dry Specific Gravity(g/cm^3)
Timbercare Aqua	30	Control	0.37	0.35
		30	0.39	0.36
		40	0.40	0.38
		50	0.43	0.41
		60	0.42	0.40

The highest air dry specific gravity is at 30 minutes and vacuum 50 minutes diffusion (0.43 g/cm^3), the lowest weight is at 30 minutes and vacuum 30 minutes diffusion (0.39 g/cm^3); the highest absolute dry specific gravity was determined at 30 minutes vacuum and 50 minutes diffusion (0.41 g/cm^3) and the lowest weight at 30 minutes vacuum and 30 minutes diffusion (0.36 g/cm^3). They reported that the main reason for the difference in density in beech wood was the different ratios of cell wall material and air void in unit volumes. They reported that the main reason for the difference in density in beech wood was the different ratios of cell wall material and air void in unit volumes (Örs & Keskin, 2001).

3.4. Adhesion Strength (N/mm^2)

The change in adhesion resistance is given in Table 4.

Table 4. Change in Adhesion Strength (N/mm^2)

Wood type	Impregnation Material (%)	Vacuum Time (Min)	Diffusion Time (Min)	Adhesion Strength (N/mm^2)	HG
Spruce wood	Timbercare Aqua	30 Dk	Control	2.43	E
			30	2.68	B
			40	2.70	A
			50	2.65	C
			60	2.61	D

HG: Homogeneity groups

The highest value in adhesion resistance was determined at 30 minutes vacuum and 40 minutes diffusion (2.70 N/mm^2) and the lowest at 30 minutes vacuum and 60 minutes diffusion (2.61 N/mm^2). Due to these properties, they can be applied in the indoor/outdoor wood industry. It is stated that the impregnating materials decrease the adhesion resistance and the samples impregnated with the long-term immersion method decrease the adhesion resistance. They found that the impregnating agents weaken the bonds between the glue layer and the surface, causing a decrease in the adhesion resistance. In the same research, higher adhesion strength

values in the bonding of wood materials impregnated with a shorter dipping method were observed. They reported that beech wood samples, which were impregnated with Imersol Aqua and sanded on the surface, and glued with polyurethane glue, gave the highest adhesion resistance as interaction (Örs *et al*., 2000). In research, the adhesion and burning properties of oak and chestnut wood material impregnated with Zinc chloride and borax were laminated with Desmodur-VTKA and PVAc adhesives, and it was stated that the impregnation process had a lowering effect on the adhesion resistance of the wood material (Okçu, 2005). In another study, beech (*Fagus orientalis*), scotch pine (*Pinus sylvestris*), linden (*Tilia perfifolia*) and chestnut (*Castanea sativa*) trees as wood material impregnated with boron components Borax, Boric Acid and Borox-Boric Acid mixture using the pressure vacuum method. They reported that the best results in bonding the samples with polimarin (Desmodur-VTKA), urea-formaldehyde, phenol-formaldehyde and PVAc glue, in bonding the linden control samples with urea-formaldehyde glue and scotch pine samples impregnated with boric acid and glued with urea-formaldehyde glue gave the best adhesion resistance in impregnated wood elements (Uysal & Kurt, 2005). In order to determine how the heat treatment affects the adhesion, it was tried to determine the adhesion resistance and the effect of heat treatment by using spruce wood panels and PE (polyethylene) adhesives. In the tests performed after the samples were prepared, they determined that the functional groups that provide the bonding on the surface were broken as a result of the heat treatment and therefore the contact angle increased; thus, they determined as a result of the mechanical tests that the bonding strength between PE (polyethylene) and wood surface is much higher than that of untreated wood material (Follrich *et al*., 2006).

4. CONCLUSION

According to the experiment results, the highest percent retention was attained at 30 minutes vacuum and 40 minutes diffusion (2.78 %), and the lowest was achieved at 30 minutes vacuum and 50 minutes diffusion (1.96 %). It has been determined that highest air dry specific gravity at 30 minutes vacuum and 50 minutes diffusion (0.43 g/cm^3), highest full dry specific gravity at 30 minutes vacuum and 50 minutes diffusion (0.41 g/cm^3), highest adhesion resistance at 30 minutes vacuum and 40 minutes diffusion (2.70 N/ mm^2), lowest adhesion resistance at 30 minutes vacuum and 60 minutes diffusion (2.61 N/mm^2). Today, we are living in a time when human wellbeing is extremely significant. It is critical for human health to treat wood with natural and environmentally friendly materials, particularly because people are constantly in contact with it. In this century, as human and environmental health is at the forefront, major joint-stock firms have been tasked with developing new preservatives in addition to R&D studies. A wide variety of strong products are available. While several product lines are designed to protect against the effects of heat, air, water, and other factors, they should be tested on a number of woods and in various time periods and the results should be shared.

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Author Contribution Statement

Hatice Ulusoy: Writing, Editing, Validation, Experiment design, Supervision. **Huseyin Peker:** Laboratory work, Statistical analysis, Validation.

Orcid
Hatice Ulusoy https://orcid.org/0000-0003-0960-3388
Huseyin Peker https://orcid.org/0000-0002-7771-6993

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Usnic Acid Inhibits Cell Proliferation and Downregulates lncRNA UCA1 Expression in Ishikawa Endometrial Cancer Cells

Mucahit Secme[1*], Yavuz Dodurga[1]

[1] Department of Medical Biology, Faculty of Medicine, Pamukkale University, Denizli, Turkey

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Abstract

Endometrial cancer is the most common gynecological cancer in developed countries. Gene expressions in cancer cells are known to be controlled by a wide array of regulatory molecules including non-coding RNAs. Noncoding RNAs contribute to many cellular events in the cancer mechanisms including proliferation, apoptosis, angiogenesis, invasion, migration and metastasis. *UCA1* has been defined as an oncogene due to its effect on cellular mechanisms such as cancer cell invasion, migration, proliferation and metastasis in different types of cancers including colon, lung, breast, bladder, endometrial gastric and pancreatic cancer. The effects of usnic acid on cell proliferation in Ishikawa endometrial cancer cells were detected by XTT assay. Total RNA was isolated by Trizol-reagent and subsequently total transcriptome cDNA was synthesized. LncRNA *UCA1* expression change between control and usnic acid treated groups was determined by the Real-Time PCR method. Usnic acid decreases cell proliferation and its IC_{50} value in Ishikawa cells was detected as 51,76 µM at 48th hour. *UCA1* expression was downregulated in usnic acid treated Ishikawa cells *in vitro*. According to results, usnic acid inhibits the proliferation of Ishikawa cells and its effect mechanisms may be associated by reducing the expression of *UCA1* oncogenic characteristic long noncoding RNA that have a role in different cellular mechanisms in cancer. In conclusion, this study may contribute further and detailed studies about usnic acid and its therapeutic potential for endometrial cancer treatment.

Corresponding Author:
Mucahit Secme, Department of Medical Biology, Faculty of Medicine, Pamukkale University, Denizli, Turkey, mehtersecme@gmail.com
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1. INTRODUCTION

Cancer is currently the second most common source of death before the age of 70 in most countries, although there have been great advances in diagnosis, treatment management and improving survival due to recent advances in technology (Bray *et al*., 2018). According to the report published by the World Health Organization, in 2018, 18.1 million people worldwide got cancer and 9.6 million people died from this disease. Furthermore, it is estimated that by 2040, these numbers will almost double (WHO, 2020). According to these statistics, cancer is seen as the biggest obstacle for increasing life expectancy all over the world in this century (Bray *et al*., 2018). A detailed understanding of the molecular mechanism involved in the pathogenesis of endometrial cancer (EC), a common gynecological malignancy, may help develop a new strategy for improving the prognosis of patients in the treatment of the disease. Research on the molecular mechanisms in endometrial cancer contributes to the development of target-specific therapies, investigation of new therapeutic agents and the identification of new biomarkers (Rizner, 2016; Lee *et al*., 2017).

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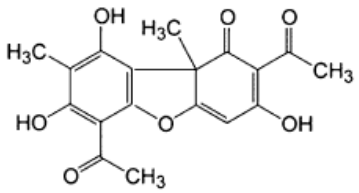
The mammalian genome is widely replicated and most of the mammalian transcriptome is composed of non-coding RNAs (ncRNAs) which are categorized as long non-coding RNA (lncRNAs) and short non-coding RNAs (sncRNAs) according to their length (Djebali *et al*., 2012). LncRNAs are a group of non-coding transcripts of >200 nucleotides (Cheetham *et al*., 2013). LncRNAs regulate gene expression at posttranscriptional, transcriptional, epigenetic and translational levels and play role in intercellular interactions, carcinogenesis, protein complexes arrangements, metastasis, invasion and angiogenesis mechanisms (Moran *et al*., 2012; Cheetham *et al*., 2013; Geisler & Coller, 2013). Urothelial cancer associated 1 (*UCA1*) is a lncRNA that was first discovered with upregulated expression in bladder carcinoma (Wang *et al*., 2006). *UCA1* is located on chromosome 19p13.12 and consists of 3 isoforms (1.4, 2.2 and 2.7 kb) with a TATA box at its 5′ end and a polyA tail at the 3′ end (Xue *et al*., 2016). *UCA1* has been defined as an oncogene due to its effect on cellular mechanisms such as cell invasion, migration, proliferation and metastasis in various cancers including colon, bladder, gastric and pancreatic cancer (Han *et al*., 2014; Shang *et al*., 2016; Zhang *et al*., 2018). Although the role of *UCA1* in the development of endometrial cancer is currently not fully elucidated. Lu *et al*. (2016) reported that *UCA1* is upregulated in endometrial cancer tissues compared to normal tissues. They also showed that increased *UCA1* expression was associated with the development of endometrial cancer and worsening outcomes in patients and *UCA1* could participate in the metastasis of endometrial cancer cells (Lu *et al*., 2016). In another study, it has been shown that *UCA1* contributes to the development of endometrial cancer by regulating the expression of the *KLF5* and *RXFP1* genes (Liu *et al*., 2020).

Natural and bioactive compounds isolated from plants, fungi and lichens have been used in the treatment of various diseases, including cancer, for many years. These natural products have attracted considerable attention from the scientific community for potential cancer treatments due to their strong and unique anti-cancer activity, minimal side effects, low toxicity, abundance inherently and low cost (Ingólfsdóttir, 2002; Nahata, 2017; Dinçsoy & Duman, 2017; Blowman *et al*., 2018; Değerli *et al*., 2020). Usnic acid (UA, $C_{18}H_{16}O_7$, 344.32 g/mol, Figure 1) a dibenzofuran derivative, is an important lichen secondary metabolite and is one of the most studied molecules in pharmaceutical research due to its anti-viral, anti-microbial, anti-proliferative, anti-cancer and antioxidant effects (Cocchietto *et al*., 2002; Ingólfsdóttir, 2002; Zuo *et al*., 2015). Recently, the anti-cancer effects of usnic acid on different cancer cells such as breast, colon, liver, ovarian, lung and gastric cancer cells have been demonstrated in various studies. In these studies, it has been reported that the possible action mechanisms of usnic acid occur through cellular events such as the inhibition of cancer cell proliferation, induction of apoptosis and cell cycle arrest, and regulation of genes and miRNAs involved in various intracellular signaling pathways (Cocchietto *et al*., 2002; Backorova *et al*., 2011; Zuo *et al*., 2015; Yang *et al*., 2016; Dinçsoy & Duman., 2017; Blowman *et al*., 2018; Değerli *et al*., 2020). Despite all these studies, there is no study on the action mechanism of usnic acid via long non-coding RNAs in cancer. In this context, the aim of this study is to determine the dose-and time-dependent antiproliferative activity of usnic acid in Ishikawa endometrial cancer cells and to elucidate the effect of usnic acid on lncRNA *UCA1* expression, which is upregulated in endometrial cancer.

Figure 1. The chemical structure of Usnic acid (Cocchietto *et al*., 2002; Ingólfsdóttir, 2002; Zuo *et al*., 2015)



2. MATERIAL and METHODS

2.1. Cell culture

Cell culture studies were performed using Ishikawa human endometrial cancer cells. Ishikawa cells were cultured at 37°C in 5% CO_2 and cells were grown in Dulbecco Modified Eagle Medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biological Industries), 20 units/ml penicillin and 20 µg/mL streptomycin, 0,1 mM amino acid solution (PAN-Biotech) and 1mM sodium pyruvate (Biological Industries). Different concentrations of usnic acid ((+)-Usnic acid, 98% HPLC, Sigma) were treated to the Ishikawa cells in time and dose-dependent manner under *in vitro* conditions.

2.2. XTT Cell Proliferation Assay

The dose and time-dependent antiproliferative efficiency of usnic acid in Ishikawa endometrial cancer cells was determined using the XTT (2,3-bis (2-methoxy-4 nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay, a colorimetric based analysis method. The study protocol was carried out in line with the instructions set by the manufacturer (Biotium cat no: 30007). Ishikawa cells were seeded into 96-well plates at a concentration of 5×10^3 cells per well. While preparing usnic acid, it was dissolved in ethanol and the solvent ratio was kept below 1: 1000. Following the adhesion of the cells, usnic acid was treated in various concentrations such as 12,5 µM, 25 µM, 50 µM, 75 µM, 150 µM during 24h and 48h. Cells not treated with usnic acid and only in the medium were used as a control group. After the usnic acid treatment incubation period, the XTT mixture was added to the medium by applying the values specified by the manufacturer. As a result of the kit incubation period, formazan formation was detected with a microplate reader (Biotek Microplate Spectrophotometer) at 450 nm (reference wavelength 630 nm) was measured spectrophotometrically. The viability (%) of Ishikawa cells was calculated using the absorbance values.

IC_{50} doses of usnic acid on Ishikawa cells were evaluated by AAT Bioquest online IC_{50} Calculator (2021) (AAT Bioquest, 2021). IC_{50} dose was used as dose group in Real-Time PCR experiment for determination *UCA1* expression change.

2.3. Total RNA Isolation, cDNA synthesis and Real Time-PCR Assay

Total RNA isolation from Ishikawa endometrial cancer cells was performed with Trizol Reagent (Hibrigen, Turkey) according to the manufacturer's instructions. Cells were planted in 6 well plates at $3x10^5$ cells. Following the adhesion of the cells, the determined IC_{50} dose of usnic acid was applied for the dose group. After the incubation, isolation steps were carried out with trizol from the control and dose groups. cDNA was synthesized using the total transcriptome cDNA Reverse Transcription Kit (abm; Cat.No. G905), according to the manufacturer's protocol. Quantitative expression analysis of *UCA1* long non-coding RNA was evaluated by Real-Time PCR (RotorGene 6000, Qiagen). *GAPDH* (house-keeping gene; glyceraldehyde-3-phosphate dehydrogenase) is used as the reference gene. The primer sequences of *UCA1* and *GAPDH* were given in Table 1. *UCA1* primer sequences were designed by Yu *et al*. (2020) and GAPDH primer sequences were designed as Yaylalı *et al*. (2016). Real-time PCR tests were performed by Real-Time RT-PCR according to the iTaq™ Universal SYBR® Green Supermix (Biorad; Cat No: 1725120) protocol. Polymerase chain reaction conditions were settled of an initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 30 s.

2.4. Statistical Analysis

All XTT and RT-PCR assays were performed in 3 replicates. The mean ± standard error was analyzed using Microsoft Excel. RT-PCR data were analyzed with the ΔΔCT method and quantitated with an online program (https://geneglobe.qiagen.com/tr/analyze/). The comparison

of the groups has been performed with "Volcano Plot" analysis, from "RT2- Profiles™PCR Array Data Analysis", which is assessed statistically using the "Student t-test". ($p < 0.05$ was evaluated as significant statistically).

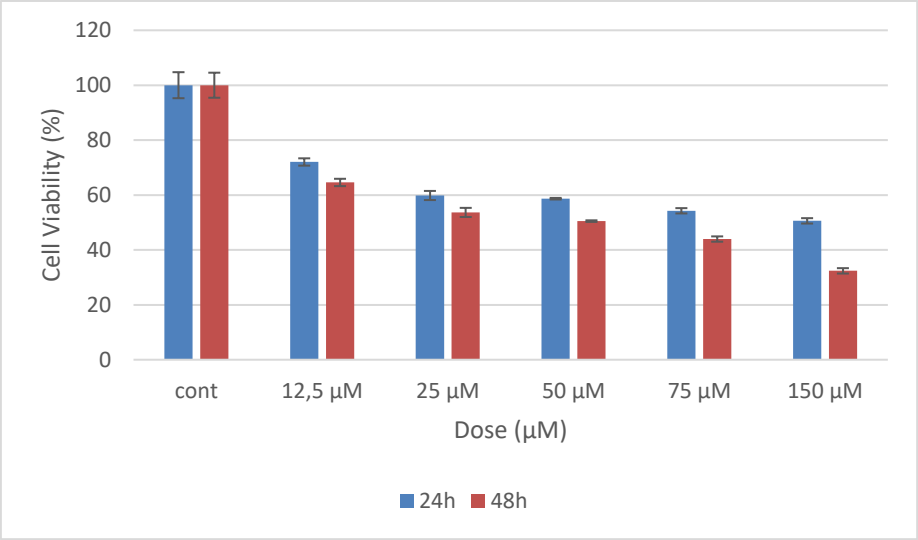
Table 1. Primer sequences of *UCA1* and GAPDH genes used in this study.

Name	Primer sequence
UCA1	F: CTCTCCTATCTCCCTTCACTGA R: CTTTGGGTTGAGGTTCCTGT
GAPDH	F: TTCTATAAATTGAGCCCGCAGCC R: CCGTTGACTCCGACCTTCAC

3. RESULTS and DISCUSSION

Anti-proliferative effects and IC_{50} dose of usnic acid were determined by using XTT assay in Ishikawa endometrial cancer cells in this study. As shown in Figure 2, usnic acid inhibited the cell proliferation of Ishikawa cells via time and dose-dependent manner. The IC_{50} dose of usnic acid was found as 51,76 µM at 48th in endometrial cancer cells using the AAT Bioquest IC_{50} calculator. When XTT results are examined, it is observed that usnic acid inhibits cell viability in Ishikawa cells due to the increasing dose in the first 24 hours, but this rate does not decrease below 50% in the dose range studied. At the end of the 48th hour, the decrease in cell proliferation was observed more clearly. This result shows us that usnic acid reduces cell proliferation further depending on the time in the dose range studied.

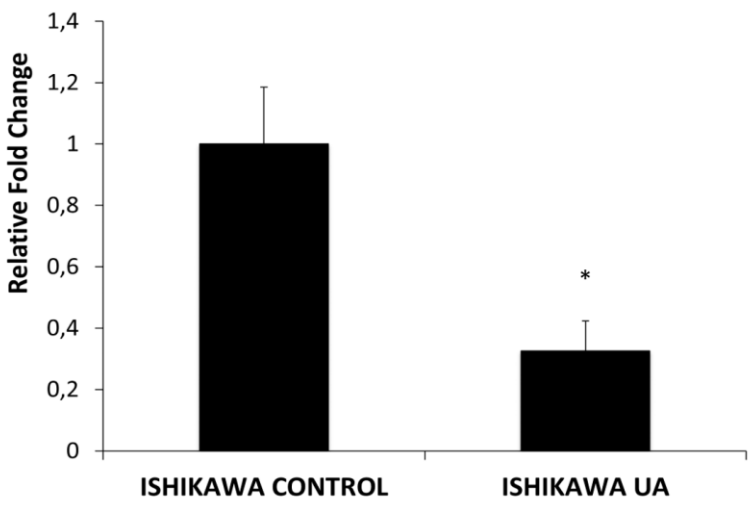
Figure 2. Usnic acid decreases cell proliferation in Ishikawa cells. Cytotoxic effects of usnic acid between 12,5 µM and 150 µM dose range in Ishikawa cells were determined by XTT assay and IC_{50} dose of usnic acid in Ishikawa cells was found as 51,76 µM at 48th hours. Absorbance data were obtained from three independent experiments.



In the literature, the anti-proliferative activities of usnic acid in different cancer cells were evaluated. Zuo *et al*. (2015) have demonstrated that usnic acid inhibited cell survival in MCF-7, MDA-MB-231 and SK-BR-3 breast cancer cell lines via inducing apoptotic mechanism via ROS-dependent mitochondrial pathway under *in vitro* conditions. They have reported that IC_{50} values of usnic acid were determined as 34.1 µM, 38.4 µM and 48.07 µM in MCF-7, MDA-MB-231 and SK-BR-3 cells, respectively. These results have supported that usnic acid decreases cell proliferation in breast cancer cells in a time- and concentration-dependent manner (Zuo *et al*., 2015). In another study conducted in breast cancer, it was shown that usnic acid

similarly decreased cell proliferation and induced apoptosis in BT-474 breast cancer cells through regulating of BCL2 and miR-185-5p (Değerli *et al*., 2020). Singh *et al*. (2013) have demonstrated that (+)-usnic acid has strong anti-proliferative activity and suppression ability on clonal growth in A549 lung cancer cells. Furthermore, it was shown that usnic acid modulated cell cycle regulators such as CDK4 and CDK6, P21/Cip1 and Cyclin D1 and induced cell cycle arrest in G0/G1 phase. In this study, it has also been reported that usnic acid induced apoptosis via mitochondrial membrane depolarization and stimulated cleavage of PARP (Singh *et al*., 2013). Dinçsoy and Duman (2017) have comprehensively evaluated anti-proliferative effects of usnic acid at different concentrations between 6.25 µM and 400 µM during 24, 48 and 72h by MTT assay in various cell lines including Hep2C human cervix carcinoma cells, Caco-2 human colon cancer cells, HepG2 human hepatocellular carcinoma cells, RD human rhabdomyosarcoma cells, Wehi mouse fibrosarcoma cells, L929 mouse fibroblast and VERO African green monkey kidney epithelial cells. They have found that lichen secondary metabolite usnic acid inhibits cell proliferation in these cell lines except L929 and Vero normal cells. They calculated EC_{50} values of usnic acid on the CaCo2, HepG2, Hep2C, RD, and Wehi cells as 7.05, 15.4, 21.8, 22.9, and 15.8 µM, respectively (Dinçsoy & Duman, 2017). Cytotoxic effects of (+)-usnic acid and (-)-usnic acid were investigated on V79, A549, and human lymphocyte cells by Koparal *et al*. (2006). According to MTT results, they found that (+)-usnic acid exhibited relatively more cytotoxic effects than (-)-usnic acid in A549 lung cancer cells. And also they reported that 100 µg mL–1 (+)-usnic acid and 100 and 50 µg mL–1 (-)-usnic acid concentrations induced cytotoxicity in human lymphocytes *in vitro* (Koparal *et al*., 2006). In a recent study performed with human gastric cancer cells, usnic acid inhibits cell proliferation in AGS cells between 10-50 µM dose range. Usnic acid significantly reduced cell proliferation in gastric cancer cells by 17-45%, 20-65%, and 52- 80% at 12, 24, 48h time points, respectively. Furthermore, usnic acid stimulated apoptosis by inducing ROS generation and DNA-PKcs and γ-H2A.X phosphorylation in AGS cells (Kumar *et al*., 2020). Galanty *et al*. (2017) showed the anti-proliferative effects of usnic acid on DU145 and PC3 prostate cancer cells and HTB-140 melanoma cells. They have reported that usnic acid decreased cell proliferation in PC3 and DU145 cells, but no significant anti-proliferative effect was observed in melanoma cells (Galanty *et al*., 2017). In addition to studies on these different types of cancer, we have demonstrated with this study that usnic acid also exhibits anti-proliferative activity in Ishikawa endometrial cancer cells under *in vitro* conditions. Extensive anti-cancer activity experiments on usnic acid, a lichen secondary metabolite, provide us with strong information that this compound can be used as an effective agent in cancer treatment studies, due to its strong biological activity. After determining the cytotoxic activity of usnic acid in Ishikawa endometrial cancer cells and determining the IC_{50} dose, the effect of usnic acid on the change in *lncRNA UCA1* expression was determined by Real-Time PCR. After the completion of total RNA isolation and cDNA synthesis, the PCR reaction was set up in triplicate. In the Syber-Green based reaction, the normalization process was carried out with *GAPDH*. According to Real-time PCR results, *UCA1* expression was decreased 3.07-fold in the dose group cells administered with usnic acid. It was found that usnic acid downregulated *UCA1* expression compared to control statistically (p= 0.030368). Relative fold change was given in Figure 3.

Figure 3. The change in expression of *UCA1* relative to the control group after usnic acid treatment in Ishikawa endometrial cancer cells. ("*" symbol indicates statistically significant (p = 0.030368) difference).



LncRNAs generally have low expression levels in normal cells, whereas the expression levels and functions of lncRNAs vary according to cell and tissue type, localization, and interacting partners or disease states. Indeed, the expression levels of lncRNA genes are modulated by sensitive molecular biological mechanisms and expressed in a tissue-specific and disease-specific manner (Chen *et al*., 2018; Dahariya *et al*., 2019; Hosseini et al., 2021). Dysregulated expression of lncRNAs has been reported in different cancer types such as leukemia, endometrial cancer, ovarian cancer, breast cancer, colon cancer etc. (Gibb *et al*., 2011; Prensner *et al*., 2011). *UCA1* has been reported as an oncogenic long noncoding RNA in many tumors, but the number of studies on the role of *UCA1* in endometrial cancer is limited and has been elucidated by recent research. Lu *et al*. (2016) have analyzed *UCA1* expression level in the proliferative endometrium, primary endometrial cancer tissue and lymph node metastasis tissue of endometrial cancer by qRT-PCR. They have analyzed the differences of clinicopathological characteristic data and survival data between the low and high expression group of *UCA1* in endometrial cancer patients. They have detected *UCA1* expression by qRT-PCR in 45 cases of endometrial tissues, 15 cases of proliferative endometrium and 15 cases of lymph node metastasis tissue of endometrial cancer. They have reported that the *UCA1* expression level in the endometrial cancer tissues was significantly higher than in the proliferative endometrium cases. Moreover, the *UCA1* expression level was found in lymph node metastasis tissues higher than primary tumor tissues. According to results, they suggested that *UCA1* was upregulated in endometrial tissues and a high expression level of *UCA1* may associate with a poor prognosis of endometrial cancer patients. Furthermore, they silenced the *UCA1* via siRNA transfection in HTB-111 and Ishikawa endometrial cancer cells. They showed that silencing of *UCA1* inhibited cell migration in endometrial cancer cells *in vitro* (Lu *et al*., 2016). Huang *et al*. (2019) have reported that the expression level of *UCA1* was significantly higher in ectopic endometrium tissues than that in paired eutopic endometrium tissues in most patients. Moreover, they suggested that downregulation of *UCA1* as a diagnostic and prognostic biomarker for ovarian endometriosis (Huang *et al*., 2019). In previous study, it was reported that upregulated *UCA1* expression may associate with the progression of endometrial cancer and patients' worsened outcome. In a study of 64 endometrial cancer patients, it has been shown that high *UCA1* expression is associated with lower survival. Molecular biological and mechanical study using patient tissues and three primary endometrial cancer cell lines, lncRNA *UCA1* modulates endometrial cancer development via regulating

KLF5 and *RXFP1* gene expressions (Liu *et al*., 2020). Jiang *et al*. (2021) have found that *UCA1* is related to cell death including autophagy and apoptosis in endometrial stromal cells (Jiang *et al*., 2021).

The discovery of biomarker genes known to have increased expression in cancers and their use in therapy has always been one of the researches. Their potential role in *UCA1*, like other long noncoding RNAs, is being investigated by basic and clinical studies in various cancers. Decreasing the expression of genes with oncogenic character with natural compounds or bioactive agents is among the studies targeted by scientists. In this study effects of usnic acid on *UCA1* expression change and cell proliferation endometrial cancer cells were demonstrated. First data about usnic acid and its effect on *UCA1* expression in Ishikawa cells were showed with this study.

4. CONCLUSION

In this study, *in vitro* cytotoxic effects of usnic acid were investigated in Ishikawa human endometrial cancer cells. LncRNA *UCA1* expression change with usnic acid treatment was also demonstrated. Investigating the effects of natural products obtained from organisms such as plants, fungi or lichen on cancer cells and especially the downregulation of the expression of genes or non-coding RNAs that play an oncogenic role contribute to further studies on cancer treatment. Therefore, it is thought that usnic acid can be a potential anti-cancer agent in endometrial cancer cells. However, more detailed molecular biological *in vitro* and *in vivo* studies should be performed to elucidate the molecular mechanism of anti-cancer activity of usnic acid in endometrial cancer.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Mucahit Secme: Investigation, Writing, Methodology, Editing. **Yavuz Dodurga:** Methodology, Writing, Review, Editing.

Orcid

Mucahit Secme https://orcid.org/0000-0002-2084-760X
Yavuz Dodurga https://orcid.org/0000-0002-4936-5954

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Determination of Toxic and Anthelmintic Activities of *Ornithogalum nutans* L., *Sternbergia lutea* (L.) Ker-Gawl. ex Spreng. and *Allium stylosum* O. Schwarz

Mehmet Ozgur Atay[1], Buse Ardil[1*], Mehlika Alper[1], Olcay Ceylan[2]

[1] Department of Moleculer Biology and Genetics, Faculty of Science, Muğla Sıtkı Koçman University, Muğla, Turkey
[2] Department of Biology Faculty of Science, Muğla Sıtkı Koçman University, Muğla, Turkey

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Abstract

In this study, toxic and anthelmintic activities of methanol extracts of aerial and underground parts of *Ornithogalum nutans* L., *Sternbergia lutea* (L.) Ker-Gawl. ex Spreng. and *Allium stylosum* O.Schwarz were investigated. In order to determine the anthelmintic activity, the time elapsed for the duration of paralysis and death was determined after the extracts of different concentrations (10, 20 and 30 mg/mL) were added to the *Tubifex tubifex* in petri dishes. Each concentration of *A. stylosum* aerial and underground parts extracts showed high anthelmintic activity. In addition, aerial extract of *O. nutans* at a concentration of 30 mg/mL showed high anthelmintic activity. *A. stylosum* extracts showed a higher activity than the standard anthelmintic drug. The toxic activity was determined against *Artemia salina* with brine shrimp lethality test. Among all extracts, the underground extract of *S. lutea* showed the highest activity with 0.002 mg/mL, LC_{50}, the aerial extract of *O. nutans* showed the lowest activity with 0.03 mg/mL, LC_{50}. Aerial and underground methanol extracts of *A. stylosum* showed high anthelmintic and lethal activity, but more specific studies are needed.

Corresponding Author:
Buse Ardil, Department of Moleculer Biology and Genetics, Faculty of Science, Muğla Sıtkı Koçman University, Muğla, Turkey, buseardil@gmail.com
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1. INTRODUCTION

The use of plant or plant-based products as medicine dates back to prehistoric times (Osama *et al*., 2020). Medicinal plants are natural resources that provide valuable phytochemical products that are frequently used in the treatment of various diseases (Sahgal *et al*., 2010). Therefore, using natural products and herbal medicines is an effective, beneficial, and economical way to treat different diseases (Azad *et al*., 2019). Part of traditional medicine often involves the use of crude plant extracts, which can contain a wide variety of molecules with uncertain biological effects (Sahgal *et al*., 2010). The health benefits of medicinal plants are related to their antioxidant activities and biological effects on cellular processes (Ukwade *et al*., 2020). Plants offer medicinal value due to the presence of substances such as alkaloids, essential oils, tannins, resins. These substances have a physiological effect on the human body. The use of local herbs as a source of medicine may have some negative effects mainly as a result of the lack of sufficient information about safe dosage and the presence of harmful by-products in some plants (Naher *et al*., 2019). Despite the worldwide recognition of herbal medicines and their use in different diseases, the safety of these treatments is still a major concern (Yang, 2020). More research is needed to determine the toxic effects of herbs used to treat various ailments (Ukwade *et al*., 2020). In the last decade, toxicity screening of a wider

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variety of food and plant products has been carried out using BSLT (Brine Shrimp Lethality Test), a method chosen by many researchers (Ajibola et al., 2020). The brine shrimp (*Artemia salina*) experiment has been proposed as a valid method to evaluate the toxic activity of plant extracts (Aydın *et al*., 2017; Suryawanshi *et al*., 2020). Combined with a reference standard, the brine shrimp test provides a fast, simple, and more importantly, inexpensive and reproducible bioassay (Tawaha, 2006). Helminth infections are global problems with serious social and economic repercussions (Dey & Ghosh, 2010). Helminth and parasitic worm infections affect over two million people worldwide. Anthelmintic drugs facilitate excretion from the body by killing (vermicide) or stunning (vermifuge) antistatic helminths (Verma *et al*., 2013). The resistance developed by helminth parasites to existing drugs becomes a problem to treat these diseases (Pal & Dey, 2011). The antihelmintic drugs used today mainly have little effect on microfilaricidal and adult worms (Murugamani *et al*., 2012; Hossain *et al*., 2015). In addition, various side effects such as epigastric pain, diarrhea, nausea, vomiting, headache, dizziness and allergic events have been reported in hosts after treatment with albendazole or mebendazole (Pal & Dey, 2011; Verma *et al*., 2013). Therefore, new drugs are urgently needed (Murugamani *et al*., 2012). Plant-derived drugs act as a prototype for developing more effective and less toxic drugs. Several medicinal herbs have been used to treat parasitic infections in humans and animals (Pal & Dey, 2011; Hossain *et al*., 2015). *Tubifex tubifex* shows anatomical and physiological similarities with the intestinal parasites (roundworm, annelid) of human. Due to their easy availability, *T. tubifex* is widely used for the initial evaluation of anthelmintic compounds *in vitro* (Chowdhury *et al*., 2015). *Sternbergia lutea* (L.) Ker Gawl. ex Spreng, a member of the Amaryllidaceae family, is a geophyte plant that generally spreads around the Mediterranean basin and blooms bright yellow in autumn (Gage & Wilkin, 2008; Güner *et al*., 2012). It has different pharmacological effects due to the alkaloids it contains (Berkov *et al*., 2009). Antioxidant, antimicrobial, antidiabetic, anti-inflammatory activities and total phenolic amount have been reported in the studies (Ünver *et al*., 2005; Aydın *et al*., 2015; Ağca *et al*., 2021). *Ornithogalum* L., which has more than 140 species in the world, is belonging the Hyacinthaceae family. Of the 45 *Ornithogalum* L. species found in the Flora of Turkey, 17 are endemic. In a study conducted on *Ornithogalum nutans* L., it was reported that seventeen cardenolides were isolated from its leaves and bulbs (Plancic *et al*., 2015). There are 146 *Allium* L. species in the Amaryllidaceae family and 65 of them are endemic. *Allium stylosum* O. Schwarz is a species that blooms between May and June and grows in forested openings and fields (Aydın, 2012). In this study, it was aimed to determine toxic (against *Artemia salina*) and anthelmintic (against *Tubifex tubifex*) activities of methanol extract obtained from the aerial and underground parts of *Sternbergia lutea*, *Ornithogalum nutans* and *Allium stylosum*.

2. MATERIAL and METHODS

2.1. Plant Materials and Extract Preparation

Sternbergia lutea was gathered in the Gülağzı neighborhood of Menteşe, Muğla providence, Turkey in 2020. Dr. Olcay Ceylan identified the plant sample were deposited in Muğla Sıtkı Koçman University Herbarium (MSKUH) under herbarium number of OC.2119. *Allium stylosum* was collected from Denizli in August-September in 2011. Identified the plant sample in Laboratory of Botanic of Pamukkale University. *Ornithogalum nutans* was collected from around the Denizli Babadağ district center. The aerial (flowers, leaves) and underground (tuber) parts of the collected plants were dried separately. After the dried plants were cut into small pieces, 100 mL methanol was added to the sample weighed 10 g. Each sample were kept in a shaking incubator at 55 ºC for 6 hours. The extraction mixture was separated from the residue by filtration through Whatman filter paper. The plant residue was left under the same conditions for second and third time with methanol added again and filtered. The solvent was removed from the obtained filtrate with the help of a rotary evaporator (Heidolph-Laborota

4011) (at 50 ºC). Then, the water in the extract was removed by lyophilization (Thermo Savant). The extracts obtained were stored at -20ºC (Turan & Mammadov, 2021).

2.2. Toxic Activity Assay against *Artemia salina* (Brine Shrimp Lethality Test)

Determination of the toxic activity assay of plant extracts against *A. salina* (Brine Shrimp Lethality Test (BSLT)) was made by modifying Krishnaraju *et al*. (2005) method. Methanol extract obtained from the aerial and underground parts of the plants was used. Two days before the assay, one pack of *Artemia* commercially available was added to the jar of 500 mL distilled water. An air motor was used to keep the air in the jar continuously. The aquarium was kept at 28 °C and in a bright environment. *Artemia* eggs were expected to hatch for 2 days. After 2 days 0.5 M sea saline solution was prepared. With different concentration (0.01, 0.05, 0.1, 0.5, and 1.0 mg/mL) was added to the test tubes containing 4.5 mL of saline solution. Later, 12 *Artemia* added to the tubes. After the tubes placed in the tube stand were placed in the aquarium on 24 hours for the emergence of *Artemia*. At the end of this time, living and non-living *Artemia* poured into Petri dishes were counted and noted.

2.3. Anthelmintic Activity against *Tubifex tubifex*

Anthelmitic activity assay was carried out using *T. tubifex*, which belongs to the same annelid group as the roundworm parasite. *T. tubifex* is used for the in vitro evaluation of anthelmintic compounds because of its easy availability and low cost. Six of the 1-2cm tall *T. tubifex* were placed in a Petri dish containing 20 mL of methanol extract. Test samples of the extracts were prepared in distilled water at concentrations of 10, 20, 30 mg/mL. Albendazole (2.5, 5, 10 mg/mL) was used as the reference standard. Distilled water was used as a negative control. Paralysis and death times were noted. The average duration of paralysis was noted when motion was lost or no movement was observed unless the worm was shaking vigorously. The time of death for each worm was recorded after it was determined that the worms did not move when they were shaken or stimulated from the outside (Dutta *et al*., 2012).

2.4. Statistical Analysis

In all assay, three replicates of each concentration were run simultaneously. $LC_{50\ (min)}$, $LC_{50\ (max)}$, LC_{50} and $LC_{90\ (min)}$, $LC_{90\ (max)}$, LC_{90} values were calculated for toxic activity by Probit analysis in STATPLUS Pro 5.9.8 package (2015).

3. RESULTS and DISCUSSION

In this study, *in vitro* toxic and anthelmintic activities of aerial and underground parts of methanol extracts of *S. lutea*, *O. nutans* and *A. stylosum* were investigated. Since plant extracts contain different bioactive compounds, they are responsible for different biological activities (Tiwari *et al*., 2011).

3.1. Brine Shrimp Lethality Test

Brine shrimp lethality test, which is a simple bioassay method to test the bioactivity of extracts, is often associated with its toxic and anti-tumor properties (Massele & Nshimo, 1995; Sonibare *et al*., 2011). In the brine shrimp lethality test assay, the % mortality rate gradually increases with an increase in extract concentration. In this way, the potentially toxic effects of the extracts are significantly predictable. Toxicity activity results of *S. lutea* extracts against *A. salina* are given in Table 1 and the best-fit line was obtained from the data through regression analysis in Figure 1. LC_{50} values of aerial and underground extracts were obtained from the plot of extract concentrations versus the percentage of shrimp dying. The underground extract of *S. lutea* showed the highest activity with 0.02 mg/mL, LC_{50}. Toxic activities of *O. nutans* aerial and underground extracts are presented in Table 2 and the best-fit line was obtained from the data through regression analysis in Figure 2. Underground part extracts (0.02 mg/mL, LC_{50})

showed a higher activity than the aerial part extract (0.03 mg/mL, LC_{50}). The toxic effect of *A. stylosum* extracts on *A. salina* is given in Table 3 and the best-fit line was obtained from the data through regression analysis in Figure 3. It showed higher activity with the underground extract (0.007 mg/mL, LC_{50}) than with the aerial part extract (0.014 mg/mL, LC_{50}).

Table 1. Average mortality rates (%) against *A. salina* of the concentrations of *S. lutea* at 24 h exposure and statistical values.

Concentrations	Aerial Part 24 h Later (%)	Underground Part 24 h Later (%)
0.01 mg/mL	59.09	68.79
0.05 mg/mL	77.78	72.22
0.1 mg/mL	80.05	90.30
0.5 mg/mL	91.11	91.11
1.0 mg/mL	100.00	94.44
Negative Control*	8.59	8.59
$LC_{50\ (min)\ (mg/mL)}$	0.001	-
$LC_{50\ (mg/mL)}$	0.008	0.002
$LC_{50\ (max)\ (mg/mL)}$	0.02	0.01
$LC_{90\ (min)\ (mg/mL)}$	0.12	0.15
$LC_{90\ (mg/mL)}$	0.30	0.43
$LC_{90\ (max)\ (mg/mL)}$	1.38	9.81
x^2	0.23	0.71

* Negative Control: distilled water

Table 2. Average mortality rates (%) against *A. salina* of the concentrations of *O. nutans* at 24 h exposure and statistical values.

Concentrations	Aerial Part 24 h Later (%)	Underground Part 24 h Later (%)
0.01 mg/mL	47.22	42.93
0.05 mg/mL	57.07	64.90
0.1 mg/mL	70.10	75.00
0.5 mg/mL	71.46	83.33
1.0 mg/mL	81.72	97.22
Negative Control*	2.78	2.78
$LC_{50\ (min)\ (mg/mL)}$	0.001	0.007
$LC_{50\ (mg/mL)}$	0.03	0.02
$LC_{50\ (max)\ (mg/mL)}$	0.08	0.04
$LC_{90\ (min)\ (mg/mL)}$	1.93	0.35
$LC_{90\ (mg/mL)}$	15.90	0.82
$LC_{90\ (max)\ (mg/mL)}$	>10000	4.60
x^2	0.51	0.27

* Negative Control: distilled water

Table 3. Average mortality rates (%) against *A. salina* of the concentrations of *A. stylosum* at 24 h exposure and statistical values.

Concentrations	Aerial Part 24 h Later (%)	Underground Part 24 h Later (%)
0.01 mg/mL	54.29	58.89
0.05 mg/mL	61.62	64.21
0.1 mg/mL	82.32	79.87
0.5 mg/mL	88.64	88.89
1.0 mg/mL	97.22	92.80
Negative Control*	54.29	58.89
$LC_{50\ (min)\ (mg/mL)}$	0.003	0.0002
$LC_{50\ (mg/mL)}$	0.014	0.007
$LC_{50\ (max)\ (mg/mL)}$	0.03	0.02
$LC_{90\ (min)\ (mg/mL)}$	0.24	0.42
$LC_{90\ (mg/mL)}$	0.54	1.52
$LC_{90\ (max)\ (mg/mL)}$	2.82	88.70
x^2	0.80	0.40

* Negative Control: distilled water

Figure 1. Regression curve plot of aerial (a) and underground (b) parts of *S. lutea*

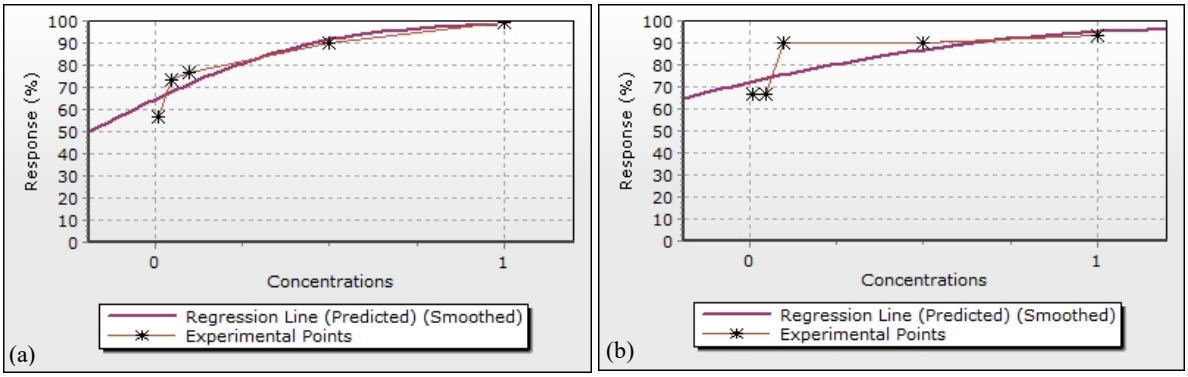


Figure 2. Regression curve plot of aerial (a) and underground (b) parts of *O. nutans*

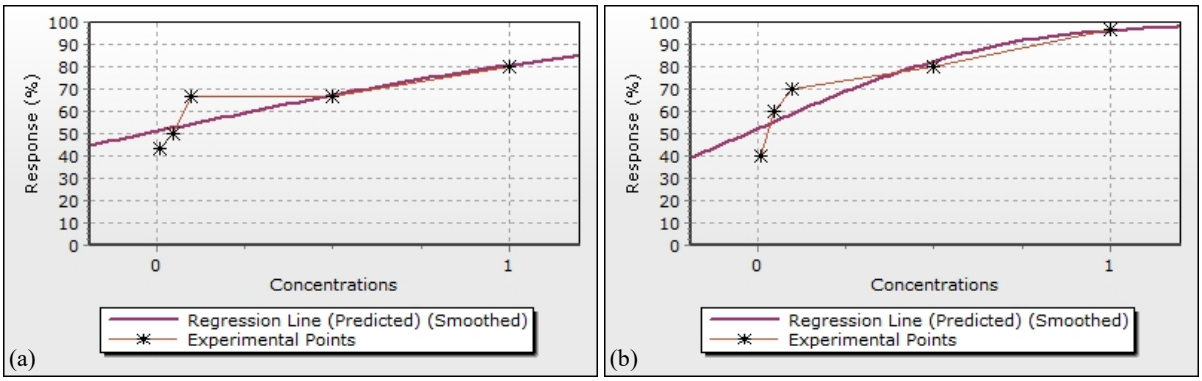
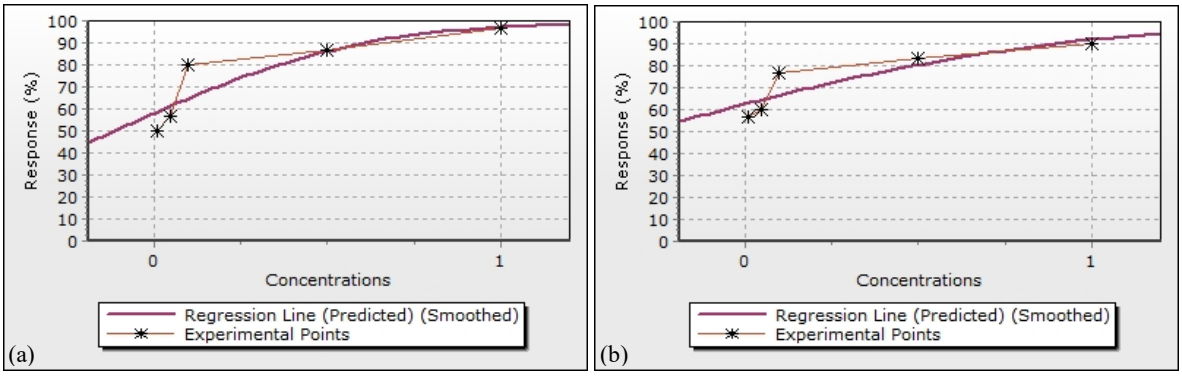


Figure 3. Regression curve plot of aerial (a) and underground (b) parts of *A. stylosum*



Among all extracts, the underground part extract of *S. lutea* (0.002 mg/mL, LC_{50}) showed the highest activity, whereas the aerial part extract of *O. nutans* showed the lowest activity (0.03 mg/mL, LC_{50}) compared to the others. It may be due to alkaloids in extracts of *S. lutea* (Evidente *et al*., 1984; Evidente, 1986). Although it is the compound responsible for the cytotoxic activity, it awaits to be discovered. There are brine shrimp lethality activity studies on some species belonging to *Amaryllidaceae* and *Asparagaceae* families. Aziz *et al*. (2014), the toxicity of methanol extracts of *Crinum latifolium* L. was determined by the brine shrimp lethality test. As a result of BSLT, the methanol extract (15,652 µg/mL, LC_{50}) exhibited lower toxicity compared to vincristine sulfate (0.839 µg/mL) used as standard. *Cordyline fruticosa* (L.) A. Chev. In a work, the toxicity of the methanol extract obtained from the leaves was investigated. In the brine shrimp lethality test, after 24 hours, the LC_{50} value was determined by counting the surviving and dying brine shrimp fry. According to the BSLT, the extract exhibited significant toxicity compared to standard vincristine sulfate (Naher *et al*., 2019). Karmakar *et al*. (2012) determined the toxicity of *Asparagus racemosus* Willd. ethanol extract by brine shrimp lethality

test. The ethanol extract showed toxic activity (10 µg/mL, LC_{50} and 47.86 µg/mL, LC_{90}). In a study, the toxicity of methanol and acetone extracts obtained from the leaves and onion parts of *Hyacinthella lineata* (Steud. ex Schult. & Schult.f.) Chouard was investigated. Its toxicity was determined by the BSLT. The methanol extract showed toxicity (4.105 ± 2.42 µg/mL) (Aydın & Mammadov, 2017). In a study, the toxicity of the ethanolic extract of the leaves of *Sansevieria trifasciata* Prain., aqua shrimp lethality test method was investigated. In toxicity tests, the ethanol extract showed potential activities depending on the dose. As a result of existing studies, they have revealed that it has toxic activity (Pinky *et al*., 2020).

3.2. Anthelmintic Activity against *Tubifex tubifex*

Effect of methanol extract from the aerial and underground parts of *O. nutans*, *A. stylosum*, and *S. lutea* on death times of *T. tubifex* are shown in Table 4. Each concentration of *A. stylosum* extracts of both aerial and underground parts and 30 mg/mL of aerial extract of *O. nutans* showed high anthelmintic activity. Death times of *T. tubifex* were shortened in all plants due to the increase in concentration. Alkaloids, tannins, terpenes, etc. can be found in plants. Bioactive compounds are responsible for anthelmintic activity (Bhadauria *et al*., 2012). In a reported study, tannins inhibit the energy production of helminths and cause death (Mute, 2009). In another study, it was shown that alkaloids cause paralysis as a result of their effect on the nervous system (Roy, 2010). Andazol® (Active ingredient: Albendazole) used as the control has been reported to cause paralysis in helminths as a result of affecting the conductivity of the muscle membranes (Martin, 1985). In a study conducted on *A. stylosum*, twenty-eight new phenolic compounds were identified, and it is known that epigallocatechin gallate has toxic effects on living organisms (Emir & Emir, 2021). These phytochemical compounds contained in the extracts may have had a similar effect on *T. tubifex*. These results are show that natural compounds of *A. stylosum* and *O. nutans* have the potential for use in the pharmaceutical industry.

Table 4. Anthelmintic activity of methanolic extracts of aerial and underground parts of studied samples

	Concentration (mg/ml)	P (min)*	D (min)**
S. lutea A.P. ***	10	64	84
	20	39	46
	30	23	35
S. lutea U.P.	10	116	203
	20	94	182
	30	98	160
O. nutans A.P.	10	92	147
	20	57	68
	30	24	34
O. nutans U.P.	10	91	124
	20	112	127
	30	55	97
A. stylosum A.P.	10	2	4
	20	2	4
	30	1	3
A. stylosum U.P.	10	25	27
	20	21	26
	30	21	25
Positive Control****	10	13	32
Negative Control*****	-	-	-

*P=Time taken for paralysis. **D=Time taken for death of worms. ***A.P.: Aerial Part, U.P.: Underground Part. **** Positive Control: Andazol® *****Negative Control: distilled water

Turan and Mammadov (2018) studied the anthelmintic activities of leaf and tuber ethanol extracts of *Cyclamen alpinum* Dammann ex. Springer. They revealed that tuber (0.52 mg/mL, LC_{50}) extract was more active than leaf extract (1.32 mg/mL, LC_{50}). Hossain *et al*. (2015) investigated the effects of 2.5, 5, 10, 20 mg/mL concentrations of methanol and water extracts of *Hopea odorata* Roxb. leaves on *T. tubifex* and determined their anthelmint activity. Levamisole (3.3 ± 0.38 min and 6.5 ± 0.76 min) was used as a standard to compare the duration of paralysis and death. A concentration of 20 mg/mL (4.05 ± 0.35 min, 7.5 ± 0.38 min) showed the closest anthelmintic activity to the standard. In this study, the anthelmintic activities of different concentrations of methanol extracts of *Sesamum indicum* L. and *Capsicum frutescens* L. were investigated. Levamisole (1 mg/mL) had the closest anthelmintic activity at concentrations of 10 mg/mL (Kamal *et al*., 2015). Das *et al*. (2011) investigated the anthelmintic activities (*T. tubifex, Pheretima posthuma*) of ethanol and water extracts of *Tamarindus indica* Linn bark and leaves. Piperazine citrate was used to compare paralysis and death times. The water and methanol extracts obtained from the shell part showed an activity close to the standard. The anthelmintic activity of different concentrations (25, 50, 100 mg/mL) of the methanol extract obtained from the tubers of *Amorphophallus paeoniifolius* (Dennst.) Nicolson was determined by comparing it with piperazine citrate. The concentration showed the most significant anthelmintic activity of 100 mg/mL (Dey & Ghosh, 2010).

4. CONCLUSION

A. stylosum showed both anthelmintic activity and brine shrimp lethal effect. The limitations of anthelmintic activity and brine shrimp experiments, such as the lack of data linking activity in experiments to *in vivo* activity, limit the value of these techniques. Other methods, such as cell line cytotoxicity assays, maybe a more useful indicator of toxic effects in vivo. Since toxicity can be associated with pharmacological activity at lower doses, plants containing toxic components may have beneficial biological activities. As a result of this study, more studies are required to isolate biologically active compounds from these plants that have anthelmintic and lethality activity.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Mehmet Ozgur Atay: Writing, Editing, Validation. **Buse Ardil:** Laboratory woks. **Mehlika Alper:** Experiment design, Supervision, Statistical analysis, validation. **Olcay Ceylan:** Experiment design, validation.

Orcid

Mehmet Ozgur Atay https://orcid.org/0000-0002-3627-448X
Buse Ardil https://orcid.org/0000-0001-6269-5133
Mehlika Alper https://orcid.org/0000-0001-6193-346X
Olcay Ceylan https://orcid.org/0000-0002-4435-8405

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Molecular, Biological and Content Studies on *Colchicum* L. Species

Beria Ozcakir[1], Mehmet Ozgur Atay[1*], Birsen Atli[1], Erkan Usluer[1], Sinan Hascelik[1], Bihter Begum Ozkilic[1], Akgul Rakhimzhanova[2]

[1] Department of Moleculer Biology and Genetics, Faculty of Science, Muğla Sıtkı Koçman University, Muğla, Turkey
[2] Kazakh Humanitarian Juridical Innovative University, Kazakhstan

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Abstract

Colchicaceae family of the genus *Colchicum* L. is in the 39 taxa present in Turkey which 18 are endemic. The Colchicine compound found in *Colchicum* species was obtained in the past, but its structure was not fully found. In the following years, this substance was developed further and reached today and was used in the treatment of diseases such as gout, rheumatism, asthma and dysentery. These species were investigated in terms of phylogenetic analysis, phytochemical content and biological activities. In many phytochemical studies, there are studies in which colchicine and its derivative compounds, which are alkaloids, have been isolated. Many antioxidant and total secondary metabolite quantification experiments such as DPPH free radical scavenging activity, metal chelation, FRAP, CUPRAC, determination of total phenolic assays were carried out on different solvent extracts of *Colchicum* species. In studies using extracts of these species, some observed that it has antioxidant enzyme inhibitory activity. The antimicrobial activities of the extracts tested on different bacterial strains have been reported. Apart from these studies, different cell lines were used on different strains of this species and their cytotoxic results were compared. The effects of colchicine obtained from *Colchicum* species on various diseases were investigated. In this review, detailed information is given about phylogenetic analysis, phytochemical, pharmacological and biological activity (antioxidant, antioxidant enzyme inhibitor, anti-inflammatory, antimicrobial, cytotoxic) studies.

Corresponding Author:
Mehmet Ozgur Atay, Department of Moleculer Biology and Genetics, Faculty of Science, Muğla Sıtkı Koçman University, Muğla, Turkey, mozgur4848@gmail.com
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1. INTRODUCTION

Plants have been used for medicinal purposes since the earliest times. Turkey has an important plant diversity because of its geographical location. *Colchicum* L. geophyte plant species, Turkey will contribute to the evaluation of the plant has been important in this review. Endemic species unique to Turkey is an important part of plant diversity. Belonging to Colchicaceae is present in Turkey 39 taxa including 18 endemics (Tüyel *et al*., 2020). *Colchicum* L. (Colchicaceae), which is popularly named by different names such as Aliöksüz, Öksüzali, Öksüzoğlan, Kar Çiçeği, Güzçiğdemi, Göçkovan, Kalkgit, Vargit and Morca, belongs to the Colchicaceae family (Baytop, 1994; Kaya, 2011). Plants belonging to the Colchicaceae family have been used as ornamental plants in parks and gardens due to their medicinal and aromatic properties as well as their elegant and beautiful appearance (Metin *et al*., 2014). Members of this family comprise perennial plants with six leaves, hypogene flowers and specialized underground structures (Kılıç *et al*., 2014). Underground structures are lumpy, corm

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and stony. The tubers are wrapped in tunica and the tunica runs along with the tuber above the ground. The leaves are flat or lanceolate and disappear after the seeds ripen. The flowers are generally purplish-pink, pink and white. Some of the blooming periods of this species take place in spring and some in the fall period (Sütlüpınar, 1981). *Colchicum* is a very important plant used to treat diseases in medicine, thanks to the tropolone alkaloid it contains. There is even evidence that *Colchicum* is used in medicine (Tüyel, 2015). Colchicine was obtained in 1820. However, its exact structure was not discovered until 1950. In the past, colchicine has been used in the treatment of diseases such as gout, rheumatism, asthma and dysentery (Düşen & Sümbül, 2007; Kılıç *et al*., 2014; Suica-Bunghez *et al*., 2017; Ahmad, 2010; Gökel *et al*., 2000). In today's modern medicine, *Colchicum* tubers and seeds were used as a source of therapeutically active alkaloids called colchicinoids. Colchicine is the main alkaloid derivative obtained from all species included in the *Colchicum* genus (Ondra *et al*., 1995). In this review, information will be given about phylogenetic, chemical compounds, pharmacological and biological activity studies based on the results of phytochemical studies on *Colchicum* species.

1.2. Molecular, Biological and Content Lighting Studies

1.2.1. Phylogenetic Studies

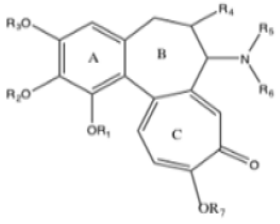
Turkey collected from 14 AFLP technology was used to examine the phylogenetic relationships between taxa *Colchicum*. A combination of 5 pairs of primers were used for this method. Later, NTSYS 2.1 software was used for statistical analysis of the data set. The phylogenetic tree was created using the neighbor-joining method and the maximum thrift method. These analyzes divided the samples into three main branches. These two analyzes have led to similar topologies. Similarly, 14 phylogenetic trees supporting taxonomy were formed through grouped main components. Using AFLP analysis, 14 taxa were evaluated using a combination of 5 primers. At the end of the study, 834 polymorphic bands were scored. The fit correlation coefficient between the data matrix and the fit matrix of the AFLP data was found to be 0.72. All participants based on molecular studies on genetic diversity, according to Turkey's other countries that have emerged, include more taxa (Metin *et al*., 2014). Sixteen *Colchicum* species and 37 different genetic variation patterns were evaluated using RAPD markers and trnL-trnF chloroplast DNA sequences together. At the end of the RAPD analysis, there were 861 polymorphic alleles containing an average of 33.88 ± 3.80 alleles for each primary gene, and also the mean frequency of the major alleles was found to be 0.067 ± 0.05. The sequence length of trnL-trnF varied between 1022 bp and 1081 bp. When the results were examined, it was seen that *Colchicum* species were grouped well because of RAPD analysis. These data were supported using PCA, structure analysis and haplotype network analysis. As a result of this study, it was revealed that this method is not sufficient to correctly distinguish and characterize the *Colchicum* genus (Tüyel *et al*., 2020). Phylogenetic analysis of 49 *Colchicum* species among 168 genotypes was performed using PCR-based markers. Using randomly amplified polymorphic DNA (RAPD), simple sequence repeat spacer region (ISSR), amplified fragment length polymorphism (AFLP) markers, 8459 alleles were identified. Besides genetic diversity analysis, principal component analysis (PCA) was also performed. As a result of PCA, it was seen that RAPD and ISSR markers can divide the population into 3 groups, AFLP markers can be divided into 5 groups, and there is no grouping because of morphological features. In this study, 4 different "neighbor-joining" clodograms were created with RAPD, AFLP and ISSR markers. Since similar results could not be found in this clodogram, a consensus clodogram covering all markers was created. This consensus showed that *C. serpentinum* Woronow ex Miscz. and *C. hirsutum* Stef. species are the furthest from each other in the branch diagram (Tüyel, 2015). Species and taxa of this kind in Turkey, non-coding regions of the chloroplast genome of the genus *Colchicum* have been copied by PCR and the transcribed region was sequenced in the "ABI 310 Genetic Analysis System". The ClustalW

program was used to compare the data obtained as a result of sequencing. The genotypes collected from these samples were determined by using STRUCTURE (v.2.3.) Software. The GenAlEx (v.6.41) program was used for principal component analysis (PCA) to reveal the genetic variation of the *Colchicum* genotype in the gene bank. For systematic evolution analysis, a neighbour-joining based genetic tree was created using the MEGA 6.06 program. STRUCTURE, PCA and phylogenetic analysis demonstrated large-scale agreement. Analysis divided the genotypes into two groups. The biggest difference between *C. kurdicum* (Bornm.) Stef. and *C. variegatum* L. populations were found to be 0.023 (Uncuoğlu, 2015). Phylogenetic analysis of ITS sequences of nuclear DNA *Colchicum* species of flora has been carried out in Turkey. They performed the sequence analysis in the "ABI 310 Genetic Analysis System". ClustalW program was used to compare the received sequence data. STRUCTURE (v.2.3.) software was used to determine the general structure and GenAlEx (v.6.41) program was used to reveal the variation of *Colchicum* haplotype in the gene pool. Then they performed principal component analysis (PCA). It has been found that this type of phylogenetic tree is fully consistent with the results of PCA and STRUCTURE analysis (Seren, 2015).

1.2.2. Phytochemical Studies

The compounds isolated as a result of phytochemical studies on *Colchicum* species and whose structures were illuminated were given by grouping. They used liquid chromatography-mass spectroscopy (LC-MS) and liquid chromatography-ultraviolet / visible photodiode array (LC-UV / Vis PDA) techniques as content analysis studies in *C. tauri* Siehe ex Stef., *C. stevenii* Kunth. and *C. tunicatum* Feinbrun species. With these techniques, they isolated 16 alkaloid compounds (Demecolcine, *N*-Methyl-(-)-demecolcine, 3-Dimethyl-*N*-methyl-(-)-Demecolcine, 2-Dimethyl-(-)-colchicine, Colchiciline, Colchifoline, Colchicine *β*-Lumi-(-)-colchicine, 3-Demethyl-(-)-demecolcine, 2-Dimethyl-(-)-demecolcine, 3-Dimethyl-(-)-colchicine, Apigenin, Isoandrocymbine, Crocifl orinone, *O*-Methyl-(-)-androcymbine, Cornigerine, Colchicine) from the underground and aboveground parts of the plants and made the structure determinations of these compounds (Gharaibeh et al., 2012). They determined the isolation and the concentration of alkaloid and colchicine (Colchicine, colchifoline, 2-demethylcolchicine, demecolcine, 4-hydroxycolchicine and N-deacetyl-N-formylcolchicine) from different parts of *C. chalcedonicum* Azn. and *C. micranthum* Boiss. species by the HPLC method. They investigated the phenolic compounds of each extract by the LC- MS / MS method. All *C. micranthum* extract has the highest amount of colchicine of all extracts. They showed that 19 phenolic compounds were present by the LC- MS/MS analysis method (Figure 1) (Gülsoy-Toplan *et al*., 2018).

Figure 1. Six compounds isolated from *C. chalcedonicum* and *C. micranthum*

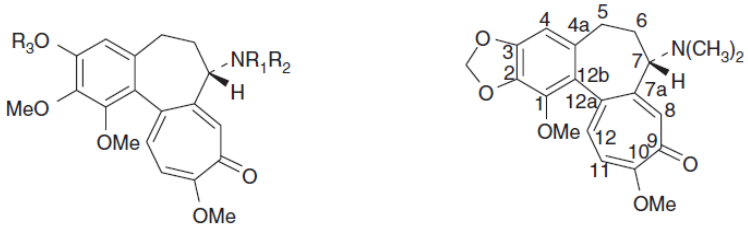


1: R_1: CH_3, R_2: CH_3, R_3: CH3, R4: H, R_5:H, R_6: $COCH_3$, R_7: CH_3
2: R_1: CH_3, R_2: CH_3, R_3: CH_3, R_4: H, R_5:H, R_6: $COCH_2OH$, R_7: CH_3
3: R_1: CH_3, R_2: H, R_3: CH_3, R_4: H, R_5:H, R_6: $COCH_3$, R_7: CH_3
4: R_1: CH_3, R_2: CH_3, R_3: CH_3, R_4: H, R_5:H, R_6: CHO, R_7: CH_3
5: R_1: CH_3, R_2: CH_3, R3: CH_3, R_4: OH, R_5:H, R_6: $COCH_3$, R_7: CH_3
6: R_1: CH_3, R_2: CH_3, R_3: CH_3, R_4: H, R_5:H, R_6: CH_3, R_7: CH_3

They identified bioactive compounds of underground and surface extracts of *C. autumnale* L. species using GC-MS and UHPLC-Q Exactive Orbitrap methods. As a result of phytochemical studies, they proved the existence of phenols, flavonoids, glycosides and terpenoids (Hailu *et al*., 2021). The content analysis of *C. crocifolium* Boiss. species was examined by combining LC-MS and LC - UV / Vis PDA methods. This dereplication strategy used UV / PDA spectra to classify compounds into one of four structural groups and combined

this with retention time-mass spectra and molecular weight to identify the compounds. This strategy uses 10 compounds known from four different structural groups ((-) -demecolcine, 2-dimethyl- (-) - colchicine or 3-dimethyl - (-) - colchicine, N-diacetyl - (-) - colchicine, (-) - colchiciline, (-) - colchicine, β-lumidemecolcine, 2-dimethyl-β-lumicolchicine or 3-dimethyl-β-lumicolchicine, N, N-dimethyl-N-diacetyl-β-lumicornigerine, (-) - isoandrocymbine and (-) - autumnaline) was applied on a small amount of *C. crocifolium* extract to copy. In addition, a new compound, N, N-dimethyl-N-diacetyl - (-) - kornigerin has been identified (Alali *et al*., 2008). They analyzed using UV - VIS, FTIR and RAMAN spectroscopy to characterize the phytochemical compound determinations (polyphenols, tannins, flavonoids, terpenoids) of the hydroalcoholic extracts obtained from the underground and aboveground parts of the *C. autumnale* plant. As a result of the photosynthesis of the extracts, the presence of colchicine, polyphenols and flavonoids was confirmed by spectral studies (UV-VIS, FTIR and RAMAN) (Suica-Bunghez *et al*., 2017). The content of the *C. baytopiorum* C.D. Brickell species was studied for the first time. Nine known alkaloids have been isolated and determined the structures by spectral methods (UV, IR, 1H-NMR and ESI / MS). In addition, the presence of three alkaloids that could not be isolated from the plant was determined by LC / MS / MS spectrometry. Phenolic acids were detailed using LC / MS and 11 phenolic acids were detected (Pırıldar *et al*., 2010). It was analyzed using LC-MS and LC-PDA systems to amplify 10 known alkaloids from the *C. crocifolium* plant. This system screen information from one of the four main group of colchicinoids identified. A new colchicinoid has been identified as N, N-dimethyl-N-diacetyl- (-) - kornigerin and with four but known compounds new to this species ((-) - colchicine, (-) -demecolcine, (-) - N-methyl - (-) - deccolin and 3-dimethyl- N - methyl - (-) - dememoline) has been isolated together (Figure 2) (Alali *et al*., 2010).

Figure 2. *Colchicum crocifolium* Boiss. isolated compounds



1: R_1=H, R_2=$COCH_3$, R_3=CH_3
2: R_1=H, R_2=CH_3, R_3=CH_3
3: R_1=CH_3, R_2=CH_3, R_3=CH_3
4: R_1=CH_3, R_2=CH_3, R_3=H

5

Ultra-high performance liquid chromatography combined with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) of extracts from flower, tuber and leaf parts of *C. triphyllum* Kunze species, mainly including alkaloids, flavonoids, lignans, phenolic acids and tyrosol equivalents It has allowed the hypothetical explanation of 285 compounds while considering different matrix extracts. Of the most abundant polyphenols, flavonoids (119 compounds) were, while the isomers of colchicine, decolcin, and lumicolychicin were some of the most common alkaloids in every extract analyzed (Senizza *et al*., 2020). They determined the alkaloid content of the bulbs, leaves and seeds of *C. turcicum* Janka (3-dimethylcolchicine, colchipholine, N-diacetyl-N-formylcolchicine, colchicine, cornigerine, 2-dimethyldemecolcine, 3-dimethyldemecolcine, demetcholine) by the HPLC method. They found that onions also contain β-lumicolchicine, luteolin and vanillic acid (Husek *et al*., 1990). The phytochemical profiles (total tropolone alkaloids, total phenolic, total tannins, total flavonoids) of *C. speciosum* Steven*, C. robustum* (Bunge) Stef*., C. autumnale* species were determined using acidic potassium dichromate, Folin-Ciocalteu and aluminum chloride

methods. In addition, the HPLC method has been used for the identification and quantitation of tropolone alkaloids. They showed that the highest tropolone alkaloid, phenolic compound, tannin and flavonoid levels were in *C. autumnale, C. speciosum* and *C. robustum*, respectively. By HPLC analysis, they revealed the presence of colchicine, dimethyl colchicine, 2-dimethyl colchicine, 3-dimethyl colchicine, colchicoside, colchipholine, corniger and N-diacetyl-N-formyl colchicine in these *Colchicum* species (Davoodi *et al*., 2021). Phytochemical profiles of *C. kurdicum* species consisting of total tropolone alkaloid, total phenolic, total tannin and total flavonoid contents were determined by spectrophotometric method. They also analyzed tropolone alkaloid profiles by the HPLC method. In HPLC analysis, they determined that N-diacetyl-N-formyl colchicine, colchipholine, colchicoside and cornigerin were the most bioactive tropolone alkaloids (Azadbakht *et al*., 2020). The quantities of colchicine found in *C. stevenii* and *C. hierosolymitanum* Feibrun species were obtained using a simple TLC spectrophotometric method with an HPLC-UV method using gradient elution. They found the highest content of colchicine in *C. hierosolymitanum* bulbs, while *C. stevenii* leaves contain the highest amount of colchicine. As a source of colchicine, they found that the two strains investigated were at comparable levels to those found in *C. autumnale*, the traditional colchicine source (Alali *et al*., 2004). The determination of the content of *C. hierosolymitanum* in the onion part extracts of the species was carried out using a single quadrupole mass analyzer equipped with LC-MS, (+)-APCI ionizing interface. (-)-Colchicine (7) and eight (1-6, 8, 9) natural analogs have been identified and identified in the alkaloid-rich fraction. These compounds were first reported for the *C. hierosolymitanum* species (Alali & El-Alali., 2005). Biologically active components of *C. hierosolymitanum* and *C. tunicatum* were investigated. Five and four known colchicinoids were isolated (HPLC) and characterized (1D-NMR, low-resolution EI-MS and APCIMS) *from C. tunicatum* and *C. hierosolymitanum*, respectively. Colchicinoids from *C. tunicatum*; those obtained from (-)-colchicine, 3-dimethyl-(-)-colchicine, (-)-cornigerin, β-lumicolchicine, (-)-androbipheniline and *C. hierosolymitanum*; it has been reported as (-)-colchicine (I), 2-dimethyl-(-)-colchicine, (-)-kornigerini β lumicolchicine (Figure 3) (Alali *et al*., 2006).

Isolation (HPLC) and characterization (H-NMR) of active ingredients of *C. stevenii* resulted in the isolation of six cytotoxic compounds. For the first time of this type (-)-colchicine, 2-dimethyl-(-)-colchicine, (-)-cornigerin, β-lumicochicine, (-)-isoandrocymbine and (-)-O-methylenedrochimbin compounds have been reported (Figure 4) (Al-Mahmoud *et al*., 2006). Nine alkaloids (3-demethylcolchicine, 2-demethylcolchicine, colchifoline, N-diacetyl-N-formylcolchicine, colchicine, cornigerine, 2-dimethyldemecolcine, 3-dimethyldemecolcine) were isolated from 7 *Colchicum* species using a high performance liquid chromatographic method. They determined the compound containing 20 phenolics from 5 *Colchicum* species. Colchicum's major alkaloid is colchicine. All parts of *Colchicum* species were shown to contain colchicine, but seeds and bulbs contained more colchicine than other plant parts (Ondra *et al*., 1995). Comprehensive (poly)-phenolic and alkaloid profiles of flower, tuber and leaf extracts (methanol, water) obtained from *C. szovitsii* Fisch. & C. A. Mey. following different extraction methods were made. In this context, ultra-high performance liquid chromatography methods combined with UHPLC-QTOF-MS (quadrupole time-of-flight mass spectrometry) were carried out. The 195 polyphenols and 87 alkaloids were described hypothetically. While the most abundant polyphenols were flavonoids (83 compounds), colchicine and 2-dimethylcolchicine were some of the most common alkaloids in every extract analyzed. It has been stated that leaf extracts are a rich source in terms of total polyphenols and total alkaloids (Rocchetti *et al*., 2019).

Figure 3. Compounds isolated from *C. tunicatum* and *C. hierosolymitanum*

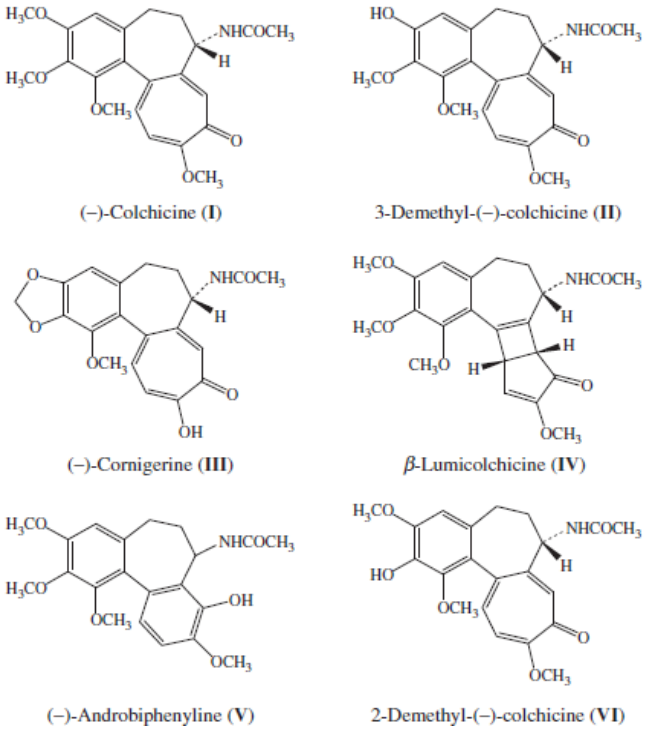
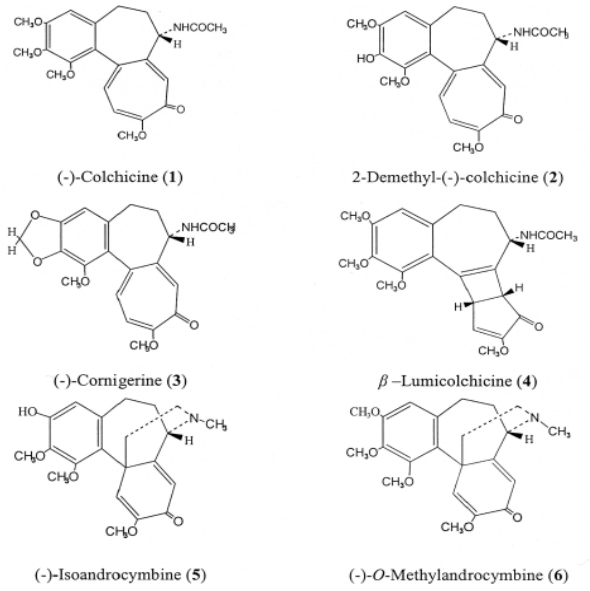


Figure 4. *Colchicum stevenii* Kunth isolated compounds



1.2.3. Antioxidant Activity Studies

In this regard, DPPH free radical scavenging activity of methanol extracts obtained from bulbs and seeds of plants was investigated in a study conducted on many *Colchicum* taxa. They reported that all extracts have a DPPH scavenging effect below 40% (Sevim *et al*., 2010). DPPH, hydrogen peroxide, NO scavenging activities, metal chelating activity and total flavonoid and total phenolic amounts were investigated from methanol extracts obtained from flower parts of *C. speciosum* species. It has been determined that the plant has an antioxidant

effect (Ebrahimzadeh *et al*., 2010). DPPH and metal chelating activity on water and acetone extracts of *C. turcicum* species; their total phenolic content was examined. At the same concentrations, 53% inhibition of water extract and 48% inhibition of acetone extract were reported. Also, total phenolic contents of water and acetone extracts were determined as equivalent. (Kılıç *et al*., 2014). Antioxidant activity of underground and aboveground methanol, acetone, gasoline and ethanol extracts of *C. balansae* Planch. was determined by DPPH and ß-carotene methods. As a result of the β-carotene experiment, the highest antioxidant activity was observed in aboveground ethanol extracts and the least antioxidant activity in underground gasoline extracts. When DPPH free radical scavenging activity was examined, it was found that the highest effect was in underground gasoline extract, while the lowest activity was in ethanol extracts (Mammadov *et al*., 2009). It was determined by DPPH, ferric thiocyanate and thiobarbituric acid methods that methanol extracts of *C. sanguicolle* K. Perss. species showed weak antioxidant activity (Karagöz *et al*., 2015). The DPPH free radical scavenging activity of the methanol extract obtained from the tuber and stem of *C. speciosum* Scavenger activity was determined as 24.63 (Souri *et al*., 2008). FRAP and CUPRAC experiments were carried out in the water and methanol extracts obtained from flowers, leaves and tubers of *C. triphyllum* species by different methods. Leaf extracts showed strong antioxidant activity in terms of CUPRAC and FRAP reducing power (Senizza *et al*., 2020). Antioxidant activities of methanol and water extracts obtained from flowers, leaves and tubers of *C. szovitsii* were determined by FRAP, DPPH, CUPRAC, ABTS, phosphomolybdenum and metal chelation methods. Methanolic leaf extracts showed the highest ferric reducing antioxidant power (FRAP) reducing power and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (Rocchetti *et al*., 2019). Free radical scavenging activities of methanol extracts obtained from *C. autumnale* tubers and flowers were determined by the DPPH method. It was observed that the inhibition values of the extract obtained from the flower (52.81%) were higher than the extract obtained from the tuber (34.60%) (Suica-Bunghez *et al*., 2017). In another study on *C. autumnale*, the antioxidant activity of extracts prepared with different solvents (n-hexane, dichloromethane, methanol) was investigated by DPPH and ABTS methods. It was observed that dichloromethane extracts showed the most activity in both methods (Hailu *et al*., 2021).

1.2.4. Antioxidant Enzyme Inhibitory Activity Studies

In a study conducted on many *Colchicum* taxa, the cholinesterase inhibitory activities of methanol extracts obtained from the bulbs and seeds of plants against acetylcholinesterase (AChE) and butynylcholinesterase (BChE) were investigated. The Only moderate activity of the methanolic extract of *C. variegatum* (35.50 + 2.26 %) was detected against acetylcholinesterase. *C. crocifolium* and *C. variegatum* extracts showed significant antioxidant enzyme inhibitory activity against butynylcholinesterase (Sevim *et al*., 2010). In a study conducted on *C. triphyllum*, antioxidant enzyme inhibitory activities of methanol and water extracts obtained from flowers, leaves and tubers were determined. While the methanol extract obtained by maceration was active against tyrosinase in terms of inhibition, it showed moderate inhibitory activities against AChE and α-amylase (Senizza *et al*., 2020). Antioxidant enzyme inhibitory activities of methanol and water extracts obtained from *C. szovitsii* were determined. Methanolic extract is more active in enzymatic inhibition against tyrosinase, glucosidase, and acetylcholinesterase (AChE) than water extracts (Rocchetti *et al*., 2019).

1.2.5. Antimicrobial Activity Studies

As a result of antibacterial studies on ethanol extracts of *C. balansae* species, they have shown that it has a weak effect against many bacterial strains (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Enterobacter cloacae* ATCC 23355, *Serratia marcescens* ATCC 8100, *Proteus vulgaris* ATCC 13315, *Pseudomonas*

aeruginosa ATCC 27853, *Salmonella typhimurium* ATCC 14028). They determined that the *Staphylococcus aureus* ATCC 25923 bacterial strain was more sensitive to ethanol extract. When their antimicrobial activity was compared with control antibiotics, they showed that ethanol extracts had lower antimicrobial activity (Mammadov *et al*., 2009). Antimicrobial activities (*Micrococcus luteus, Mycobacterium smegmatis, Saccharomyces cerevisiae, Aspergillus niger*) of 9 compounds isolated from *C. brachyphyllum* Boiss. & Hausskn. species were investigated. None of the isolated compounds showed any activity (Alali *et al*., 2005). Turker and Usta (2008) investigated the antimicrobial (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus pyogenes, Staphylococcus aureus, Staphylococcus epidermidis*) activity of the fresh extract of *C. szovitsii*. While the fresh extract showed low activity (7.7 ± 0.2l mm) against *S. aureus*, it did not show any activity against other bacteria. The antimicrobial activities of *C. luteum* Baker methanol extract and its different fractions against *Escherichia coli*, *Bacillus subtilis, Klebsiella pneumonae*, *Shigella flexenari*, *Staphylococcus aureus* and *Salmonella typhi* bacteria were investigated. The Methanol extract (58 %) showed the highest activity against *Bacillus subtilis* (Ahmad *et al*., 2006).

1.2.6. Cytotoxic and Anti-inflammatory Activity Studies

Anti-inflammatory activities of extracts obtained from *C. autumnale* were investigated. Insertion analysis revealed that colchicoside (3 dimethyl colchicine glucoside) inhibited IL-6 having a binding energy of -7.1 kcal / mol with an RMSD value of 0.00 (Hailu *et al*., 2021). The cytotoxic effects of pure (PC) and chitosan-loaded (CCNPs) forms of colchicine isolated from *C. autumnale* on different cancer cell lines (HeLa, SKOV3, MDAMB231, Panc-1, PC3) were investigated. When looking at IC_{50} values, they are both effective against HeLa and SKOV3 cell lines. It has been found that their cytotoxic activities are weak against MDAMB231, Panc-1 and PC3 cell lines (Uddin *et al*., 2019). Another study investigated the cytotoxic effect of different concentrations of colchicine isolated from *C. autumnale* on the MCF-7 human breast adenocarcinoma cell line. It inhibited MCF-7 viability at concentrations of 10 and 100 µg/mL (Bakar-Ateş *et al*., 2018). In a study, the effect of colchicine isolated from *C. autumnale* on gastric carcinoma (AGS, NCI-N87) was investigated. It has been determined that it inhibits the proliferation of AGS and NCI-N78 cell lines depending on the dose. It promotes apoptosis of NCI-N87 cells. The *in vivo* experiment confirms that colchicine administration significantly suppresses tumor growth in mice through the induction of apoptosis at different concentrations (0.05 and 0.1 mg / kg) (Zhang *et al*., 2019). The cytotoxic effects of colchicine isolated from *C. autumnale* on HT-29 cell lines were investigated. They determined that the vitality of the cells decreased depending on the dose. Early apoptosis has been reported when cells are treated with 1 µg / mL colchicine (Huang *et al*., 2015). *C. baytopiorum* contains colchicine and its derivatives. The effects on the viability of the HeLa cell line were investigated. It was determined that both apoptotic and autophagic regulatory gene expressions increased significantly in the treatment group compared to the control group. It has also been reported to induce the crosslink between apoptotic and autophagic cell death in HeLa cells (Özsöylemez *et al*., 2016). The cytotoxic activity of 9 compounds isolated from *C. brachyphyllum* species on cancer cell lines (MCF-7, H460, SF268, BST) was investigated. They showed general cytotoxicity against BST (Alali *et al*., 2005). Cytotoxic activities of ethanol extracts obtained from *C. pusillum* Sieber species were investigated. All concentrations of the extracts have toxic effects on Colo-320 cells. As a result of immunohistochemical staining in Colo-741 cells, β-catenin, LGR-5 and caspase-3 immunoreactivities significantly increased, while Wnt7a immunostaining intensity decreased. It has also been found that it significantly increases caspase-3 immunoreactivity, indicating that apoptotic pathways are triggered (Becer *et al*., 2019). All pure compounds, cell lines on *C. hierosolymitanum* and *C. tunicatum* species; MCF-7 human breast carcinoma, NCI-H460 human large cell lung carcinoma, and SF-268 human astrocytoma were evaluated for cytotoxicity against three human

cancers. Among the compounds defined between EC_{50} values, (-)-Colchicine (I) and (-)-cornigerin (III) was determined as the most bioactive compounds (Alali *et al*., 2006). It has been shown that colchicine extracted from plants of the *Colchicum* genus has different physiological effects in various variations. However, the effect of colchicine on cytosolic free Ca^{2+} levels and related physiology on human oral cancer cells is unknown. This study examined whether colchicine changed Ca^{2+} homeostasis. OC2 has been observed to cause cytotoxicity in human oral cancer cells (Sun *et al*., 2019). To determine the cytotoxic activity on extracts of all parts of the *C. baytopiorum* species, the toxicity of *Artemia salina* was tested. As a result of the test, the extracts showed significantly high cytotoxic activity (LC_{50}: >100–23.20 µg / mL). In addition, they determined the cytotoxic activity of the extracts with the colorimetric MTT test on the K562 and HL60 cell lines. Except for seed extracts, all methanol extracts showed more cytotoxic activity on HL60 cells than K562 cells (Pırıldar *et al*., 2010). Brine shrimp eggs (*A. salina*) were used to determine cytotoxicity in the *C. luteum* taxon methanol extract. The median lethal dose (LD_{50}) given on the shrimp was measured as 42.43 µg / mL, expressing significant cytotoxic activity. The toxic potential effect of *C. luteum* Baker's fractionation did not change much. Chloroform and n-butanol fractions expressed the same cytotoxicity with LD_{50}: 42.43 µg / mL. The ethyl acetate fraction decreased slightly to LD_{50} 43.30 µg / mL in case of cytotoxic propensity. The aqueous fraction showed the least cytotoxic activity with LD_{50} of 45.15 µg / mL in the test. (Khan *et al*., 2011).

1.2.7. Pharmacological Activity Studies

According to many researchers, acetylcholinesterase inhibitors are stated to be the most effective treatment method to treat Alzheimer's disease. In the treatment of this disease, drugs with these inhibitors are used. However, some of these drugs are suggested to have severe side effects such as diarrhea, fatigue, nausea, muscle cramps as well as gastrointestinal, cardiorespiratory, genitourinary and sleep disorders. For this reason, new acetylcholinesterase inhibitors with higher potency are sought that can be obtained naturally. *C. balansae* is a plant that may be suitable for this inhibitor (Chattipakorn *et al*., 2007; Dhivya *et al*., 2014). For over 45 years, *Colchicum* species have been studied and used for therapeutic purposes. Different types of this plant are also grown for use in the pharmaceutical industry today. Tropolone alkaloid content of the species; FMF is helpful for the treatment of diseases such as gout, amyloidosis, cirrhosis, Behçet's disease, psoriasis, Hodgkin's lymphoma, myeloid leukemia and skin cancers. Since the synthesis method of alkaloids from *Colchicum* species has not been found yet, colchicine and other alkaloids are extracted from the plant source (Toplan *et al*., 2016). *C. luteum* type is a rare, expensive, and very medicinal herb. The medicine obtained from this plant is called Suranjan Talkh. This drug: The presence of alkaloid colchicine, which is claimed to be effective in arthritis, gout, rheumatism, and is used as a carminative, laxative and aphrodisiac. It is also applied externally to relieve inflammation and pain. It is an effective drug that draws attention by defining its palliative, therapeutic and other uses (Ansari *et al*., 2020). Arthritis is one of the most common diseases in the world that affects hundreds of millions of people. Suranjan Shirin is an important Unani Medicine drug derived from *C. autumnale* and widely used in the treatment of arthritis. The onion of the *C. autumnale* species is mainly used to relieve pain and inflammation and is also used to treat acute gout and some gout infections. (Akhtar & Siddiqui, 2018). It is one of the main drugs derived from the Suranjan *Colchicum* species and used in the Unani Medical System for arthritis disease. According to the research, there are two types of the drug under the name of Suranjan; one is Suranjan Shirin from *C. autumnale* species and the other is Suranjan Talkh from *C. luteum* species. They can be confused because they are morphologically similar to each other. Therefore, the marker compound (total alkaloid content) was quantitatively determined to distinguish these species. High performance liquid chromatography (HPLC) has been applied for both drugs. Colchicine concentration was higher in Suranjan Talkh (0.21%) compared to

Suranjan Shirin (0.15%). Thus, a phytochemical concentration criterion, namely colchicine content, is presented to distinguish between Suranjan Shirin and Suranjan Talkh (Siddiqui *et al*., 2019). Colchicine is an antimitotic agent like vinca alkaloids because it binds to tubulins and disrupts microtubule polymerization. This is a known main mechanism of colchicine (Levy *et al*., 1991). In a study conducted on rats, colchicine was shown to reduce urine excretion of Tamm-Horsfall protein, altering its structure, thus preventing it from forming a complex with the Bence-Jones protein (Sanders, 1993). This condition causes acute kidney failure in myeloma patients. According to the data obtained, although colchicine administration was successful in mice, it was reported that there was no change in serum and urine levels of Tamm-Horsfall protein in healthy volunteers (Cairns *et al*., 1994). They administered colchicine and silymarin to rats with liver damage, respectively. Both compounds have been shown to exhibit similar hepatoprotective effects against chronic liver damage (Favari & Pérez-Alvarez, 1996). Colchicine was administered to rats with CCI4-induced cirrhosis for 12 months. A decrease in the formation of cirrhosis tissue was observed in all of these rats (Le Hello, 2000). Using colchicine in the treatment of Gout was approved in 1987. Compared to patients who received placebo, there was a significant decrease in complaints between 18-30 hours after administration in patients who were administered colchicine. While the complaints decreased within 24 hours, it was observed that each patient had diarrhea 24 hours ago. Colchicine has been reported to have fewer side effects than other anti-inflammatory drugs (Ahern *et al*., 1987). Patients with chronic gouty arthritis in allopurinol treatment were divided into two groups. One group received 0.6 mg colchicine twice a day, while the other group received a placebo for 3 months in a randomized double-blind study. A significant reduction in acute gout attack was observed in the first group, on both allopurinol and colchicine treatment. (Borstad *et al*., 2004). In this study, 2 groups of 10 FMF patients were administered orally 0.6 mg colchicine or placebo three times a day for 6 months. They used colchicine to suppress febrile attacks. While 59 attacks were observed in 9 patients who received a placebo treatment, this number was observed as only 2 patients with 5 attacks in patients who received colchicine treatment. These results are statistically significant ($p < 0.002$) and prove that colchicine administration is highly effective in preventing attacks (Goldstein & Schwabe, 1974). Colchicine has been suggested for the treatment of serositis in patients suffering from FMF disease. In this study, three hundred and fifty FMF patients younger than 16 years of age were treated with colchicine for 6-13 years. Based on the results of the treated patients, 64% had complete regression and 31% partial regression. Amyloidosis did not develop in the colchicine regimen and colchicine side effects (Zemer *et al*., 1991). The effect of colchicine in non-insulin-dependent diabetes mellitus (NIDDM) has been studied. They noted that when colchicine was given to NIDDM patients at a dose of 0.5 three times a day, it significantly reduced the level of fasting and postprandial glucose in the blood. This study showed that colchicine has antidiabetic properties (Das, 1993). They studied patients with chronic idiopathic constipation to determine whether colchicine could increase bowel movements. As a result of the colchicine doses given for certain periods, bowel movements, nausea, abdominal pain and bloating symptoms were observed in patients. Based on the data obtained, it was concluded that it could be an effective agent for treating constipation patients (Verne, 2003). Colchicine is widely used in Behçet's syndrome. Studies in men and women have been followed over a long period of time. As a result of these observations, it was seen that colchicine exhibits different effects according to gender, but it is effective for arthritis in both sexes. It has been stated that colchicine has a visible effect on genital lesions in female patients (Yurdakul *et al*., 2001). Twenty-two psoriatic arthritis patients were treated with colchicine at a dosage of 0.02 mg per kg per day for 2-4 months. As a result of this treatment, 8 out of 9 patients showed significant improvement (Wahba & Cohen, 1980).

4. CONCLUSION

Colchicum, 39 taxa of a large distribution area with a large majority in Turkey are endemic. There are many phylogenetic and biological activity studies on these taxa. Phytochemical, antioxidant, antioxidant enzyme inhibitor, anti-inflammatory, antimicrobial and cytotoxic activities have been demonstrated in various studies on extracts obtained from these plants with different solvents. the advanced studies that can be done on the therapeutic alkaloids they contain, it will contribute to the pure and effective use of the compounds in the plant. Studies on *Colchicum* species have shown that the plant contains dense alkaloids and colchicine is the main alkaloid obtained from all species. It is known that alkaloids have pain relief, antitumor, treatment of cardiovascular diseases, antimalarial and morphine properties. It was understood with the literature reviews that the species belonging to this genus should be examined and brought to the world of science in our country. With this review, it was concluded that *Colchicum* species are worth researching in terms of the compounds they contain and their pharmacological effects.

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The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Beria Ozcakir: Writing, Editing. **Mehmet Ozgur Atay:** Writing, Editing. **Birsen Atli:** Writing of Phylogenetic Studies Part. **Erkan Usluer:** Writing of Phylogenetic Studies Part. **Sinan Hascelik:** Writing of Cytotoxic and Anti-inflammatory Activity Studies Part. **Bihter Begum Ozkilic:** Writing. **Akgul Rakhimzhanova:** Editing.

Orcid

Beria Ozcakir https://orcid.org/0000-0001-6563-0095

Mehmet Ozgur Atay https://orcid.org/0000-0002-3627-448X

Birsen Atli https://orcid.org/0000-0003-2461-6435

Erkan Usluer https://orcid.org/0000-0001-6293-5918

Sinan Hascelik https://orcid.org/0000-0002-4567-8355

Bihter Begum Ozkilic https://orcid.org/0000-0002-6049-3594

Akgul Rakhimzhanova https://orcid.org/0000-0001-9939-2267

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